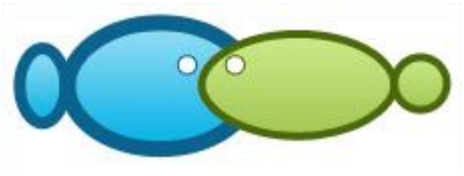

Non-specific immune response development of *Aeromonas hydrophila*-infected Nile tilapia *Oreochromis niloticus* with application of immunostimulant of saluang belum (*Luvunga sarmentosa*) extract

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Abstract. Saluang belum (*Luvunga sarmentosa*) contains secondary metabolite compounds, saponin and tannin, that possess roles in stimulating body defense or as immunostimulator. This study aims to know the effect of saluang belum extract application on non-specific immune system development of Nile tilapia through measurements of total leucocyte, phagocytotic activity, and survival rate (SR). It used complete randomized design with 4 treatments and 3 replications. Treatment applications were saluang belum with concentration of 5 g kg^{-1} feed, 10 g kg^{-1} feed, 15 g kg^{-1} feed, and without saluang belum extract as control. The fish were given saluang belum extract and after 21 days, a challenge test was carried out. The phagocytotic index and total leucocyte measurements were done on day-7, 14, 21, and 28 (7 days after the challenge test). SR was assessed at the end of the study. Results showed development of phagocytotic activity in the fish administered with saluang belum extract. The highest value was recorded at the dose of 15 g kg^{-1} feed, 34.12%, quite different from the control treatment (without addition of saluang belum immounostimulant), 17.51%. Total leucocytes were also the highest at the dose of 15 g kg^{-1} feed, 127,400 cells mm^{-3}, where the control treatment had only 52,370 cells mm^{-3}, and in day-28 or 7 days after the challenge test, total leucocytes were lower at the dose of 15 g kg^{-1} feed. The highest SR was found at the dose of 15 g kg^{-1} feed, 89.00%, whereas the control treatment had only 46.67%. Application of saluang belum extract could increase the immune response, such as phagocytotic activity, total leucocytes, survival rate, of Nile tilapia. The best dose that could give immune response was 15 g kg^{-1} feed.

Key Words: total leucocyte, phagocytotic index, survival rate, fish, body defense.

Introduction. Nile tilapia (*Oreochromis niloticus*) has future prospects due to favorable biological properties, such as easy to breed, grow fast, thick and compact meat. This species is also tolerant to various environmental conditions and has a broad response to different food sources. However, in Nile tipalia culture, there are several constraints that can influence the fish production level. One of them is bacteria-related disease infection. Bacterium that mostly infects the tropical freshwater fish is *Aeromonas hydrophila* as cause of Motile Aeromonas Septicemia (MAS). MAS or Hemorrhagic Septicemia usually infects Nile tilapia and other freshwater fish (Thune et al 1993; Austin & Adams 1996). Bacteria-induced infection disease shows the following symptoms: red mouth, reddish spotty body, inflating stomach, broken fins, weakness, rotative swimming to the surface, and loss of appetite. The loss can be very high, since it can cause massive fish mortality in relatively short time (Susanto 1988; Kordi 2004).

Fish disease usually appears in relation with weak condition of the fish due to fish handling, excessive feeding, and unfavorable environmental condition. The most efficient

mitigation effort is disease prevention through immunostiulant administration. Immunostimulant is a chemical, drug, stressor, or action that increases non-specific or innate immune response directly interacting with the cell of the innate immune response-activating system to make the animal be more resistant to infections of viruses, bacteria, fungi and parasites (Raa 2000; Syakuri et al 2003; Bond 2011). Robertsen et al (1990) added that immunostimulant is the compound that is able to stimulate body defense activity.

The working mechanism of immunostimulant in phagocytotic cell function development, as suggested by Jawetz et al (1996), is to stimulate the macrophage to produce interleukin that will activate T lymphocyte cell and B lymphocyte cell. T cell will then produce interferon to rebuild the macrophage that can phagocytize bacteria, viruses, and other alien particle entering the body. In healthy condition, immunostimulant can be utilized as preventive effort to hinder the disease and to increase endurance.The supplementary therapy here means immunostimulant is not the main medicine to fight against the disease, but it helps only increase the healing process. Nevertheless, immunostimulant will be more beneficial on condition where the body immune system declines (Farooqi et al 2018.) Various immunostimulants can be used, one of which is saluang belum *Luvunga sarmentosa*.

Saluang belum is one of the forest plants in Central Kalimantan that are beneficial as traditional drug. This species contains secondary metabolites as saponin and tannin that have antibacterial and antioxidant effect (Handayani et al 2019). Several organic compound groups, such as flavonoid, glycoside, triterpenoid, steroid, alkaloid, and some other organic compounds have a role in stimulating body defense or as immunostimulator (Syarifah 2006; Astuti et al 2017). The antibacterial testing of saluang belum on bacterium *Staphylococcus aureus* shows an inhibition zone of 26.7 mm at the concentration of 0.5%, 21.6 mm at 1%, and 20.5 mm at concentration of 5% (Qamariah et al 2018). The study was also accomplished on combination of saluang belum extract and yellow root (*Arcangelisia flava*) that has antibacterial effect on bacteria *A. hydrophila in vitro*. The effective concentration of this extract combination was found at 55 g per 150 mL^{-1} with an inhibition zone of 15 mm (Maryani et al 2020). Based on these findings, saluang belum extract needs to be studied as immunomodulator on the immune system of the Nile tilapia in order to prevent the MAS disease. This study aims to know the effect of saluang belum extract application on the non-specific immune system development through the measurements of total leucocyte, differential leucocyte, phagocytotic activity, and survival rate (SR).

Material and Method. This study was accomplished in September 2018 - January 2019 in the Laboratory of Fish Health, Department of Fisheries, Faculty of Agriculture, Palangka Raya University, whereas the extraction of saluang belum root was done in the Laboratory of Fish Quarantine Station, the Class 1 Fisheries Product Quality and Security Control, Palangka Raya, Central Kalimantan.

Assay fish. The test animals used in this study were 100 individuals of 7.5-8.5 cm long Nile tilapia taken from the fish hatchery unit in Palangka Raya. They were acclimated for about two weeks. The fish were then put into 55 x 35 x 45 cm aquarium containing 67 L of water at the density of 12 ind aq^{-1}. Water replacement was done daily as much as 20% through dirt siphoning from the bottom.

Bacteria Aeromonas hydrophila. Bacteria *A. hydrophila* were obtained from *A. hydrophila*-infected fish from local fish farmers in Palangka Raya. The bacteria *A. hydrophila* were isolated from the fish showing the symptoms of MAS disease, purified, and identified. They were cultured in Tryptone Soya Agar (TSA) medium and incubated for 24 hours at 37^{o}C. Bacterial identification was also done to ensure that the isolated bacteria were *A. hydrophila* with the following characters: round colony, convex, yellowish, and has one flaggela (monotrichous flagella) appearing from one of the polar (Laith & Najiah 2013).

Experimental feed preparation with addition of saluang belum (L. sarmentosa). Saluang belum was chopped in small pieces and blended to powder, then weighed following the treatment doses. It was put in sterile aquadest up to being submerged and put into an Erlenmeyer glass for 10 min centrifugation at 6,000 rpm to obtain supernatant. As much as 10 mL of the supernatant was then put into a sterile Petri disk and left for 24 hours (maceration) over the Laminary Air Flow (LAF) in order to reduce evaporation and obtain the desired extract. The extract was weighed using a 0.01 g digital balance as desired doses. It was then dissolved in some water (100 mL for 1 kg feed preparation) and sprayed to the feed. Saluang belum solution mixing was accomplished in such a manner so that it could evenly be mixed in the feed. Saluang belum extract-containing feed was then wind-dried at room temperature, put in the plastic bag, and stored in the refrigerator until use.

Experimental design. The study applied complete randomized design with 4 treatments and 3 replications. The treatments consisted of Control (A) – without saluang belum root extract, B – application of 5 g kg^{-1} feed, C – 10 g kg^{-1} feed, and D - application of 15 g kg^{-1} feed).

Before use, the fish were acclimated for 2 weeks in 25 L tank. The tank was cleaned by dipping in 20 ppm of $KMnO_4$ 20 ppm for 24 h, and then rinsed with clean water. During acclimation, the fish were fed with pellet as much as 2% body weight daily. Water was replaced as much as 25% daily. Physical and chemical parameters were measured and monitored weekly. Dissolved oxygen was measured using Winkler method, pH measurement used pH-meter, water temperature with thermometer, and ammonia measurement used Sera test kit.

Challenge test. After 21 days of immunostimulation, challenge test was done. This test was intended to know the fish endurance against the bacteria *A. hydrophila* after administered with saluang belum root extract. The challenge test was carried out through intramuscular injection of 0.1 mL of 10^7 mL^{-1} of *A. hydrophila* on fish dorsal muscle. The injected fish were put back into the aquarium for 24 h observation on the fish and mortality estimation.

Research parameters

Phagocitic index measurements. Phagocytotic index measurements were conducted at day-7, 14, 21, and 28 (7 days after the challenge test). Phagpcytotic activity measurements followed Anderson & Siwicki (1993) in Farouq (2011). The blood sample of Nile tilapia was taken as much as 20 µL and put into an Eppendorf flask, then added with 20 µL of *A. hydrophila* as antigen at the density of 10^7 cells mL^{-1}. The suspension was then homogenized and incubated for 20 min at room temperature. As much as 5 µL suspension was taken and made blood smeared preparate. The blood sample of the fish was dropped on the cover glass at the right side. Other object glass was also placed at the right of the blood forming 30° angle. The object glass was withdrawn to the left by constantly touching the blood up to forming sufficiently thin blood object glass that was easily observed. Afterwards, the object glass was wind-dried. The dry object glass was then fixed in methanol solution for 5-10 min and wind-dried. The object glass was then immersed in Giemsa solution for 10-15 min, rinsed in aquadest, and wind-dried. The smear object glass was observed under the microscope. The phagocytic index was estimated based on percent of phagocytic cells indicating phagocytotic process of total phagocytic cells counted.

Total leucocytes. Total leucocyte measurements followed Blaxhall & Daisley (1973). Blood sample was sucked using pipette containing white mixing thread up to scale 0.5 and added with Turk's solution up scale 11. The pipette was swung forming value-8 for 3-5 min, so that the blood was evenly mixed. Afterwards, 2 first blood drops were removed from the pipette and dropped on the haemocytometer. Counts were done on 5 large boxes of the haemocytometer. Number of the counted leucocytes was multiplied by 50 cells mm^{-3}.

Survival rate. Survival rate (SR) is the percent of fish amount living after feeding application. SR estimation was done at the end of the experiment.

Results and Discussion

Phagocytic index. Phagocytic index is the ability of the organism to phagocytize alien materials that will invade the defense system. In normal condition, number of leucocytes is positively correlated with phagocytotic activity (Sani et al 2014). In other words, the higher the number of leucocytes, the higher the phagocytotic activity. The present study found that application of salung belum extract-containing feed for 21 days was capable of developing the phagocytotic activity. After infected with bacteria *A. hydrophilla*, the phagocytotic index of all treatment groups increased. The highest phagocytotic activity after infection was recorded in the treatment of 5%. The phagocytotic index condition of Nile tilapia during the application of saluang belum immunostimulator and after the challenge test is shown in Figure 1.

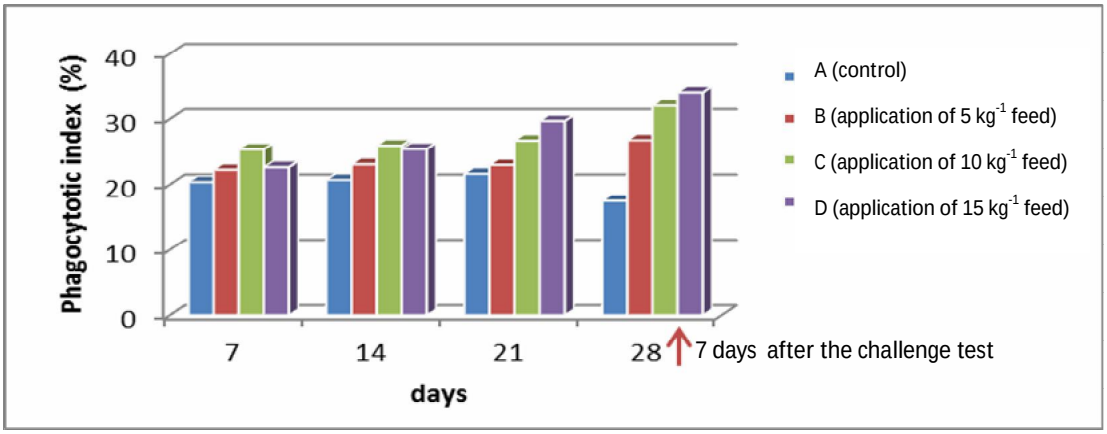

Figure 1. Phagocytotic index of *O. niloticus* during saluang belum application and after the challenge test.

Figure 1 demonstrates that application of saluang belum immunostimulant at the the doses 5-15 g kg^{-1} feed for 21 days develops the phagocytotic index. The highest index was recorded at the dose of 15 g kg^{-1} feed, 34.12%, and it is quite different from that of the control treatment, 17.51%. It is in line with Qomariyah et al (2017) that increased fish body immune could be seen from phagocytic index development. According to Johnny et al (2012), increased phagocytotic activity could occur at the initial response of the immunostimulant administration or initial infection, whereas low phagocytotic activity is caused by stress, lack of protein and vitamin, and chronic infection. The pathogen elimination and destruction process occurs through phagocytotic mechanism of the macrophage cell (Woo 1995). Phagocytosis is the initial step for the next immunity response mechanism, i.e the formation of specific response as antibody. It includes attachment, ingestion, degranulation, destruction, and antigen digestion (Bratawijaya 2002; Lukistyowati 2011). Phagocytosis starts from contact of the cell membrane with the particle (toxin) that will activate the flavoenzymatic system in nicotinamide adenine dinucleutide phosphate (NADP) oxidase membrane, then forms reactive oxygen intermediates (ROI). NADP oxidase will react and form anion superoxide (O^{-2}) that with the catalyzator superoxide dismutase (SOD) becomes hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) that is toxic to *A. hydrophila* (Galindo-Villegas & Hosokawa 2004; Abbas et al 2005). Phagocytotic index examination is useful for determination of fish health condition. According to Alifuddin (2002), blood examination is important to ensure the diagnosis of a disease. Blood components will change if the fish physiology is disturbed. Changes in fish blood components could occur quantitatively or qualitatively. Therefore, it is very important to know the fish blood condition and their health status for future disease prevention.

Total leucocytes. White blood cells in fish are part of the non-specific body defense system. Observations on total leucocytes in Nile tilapia were performed during the application of saluang belum immunostimulant and after the challenge test (Figure 2).

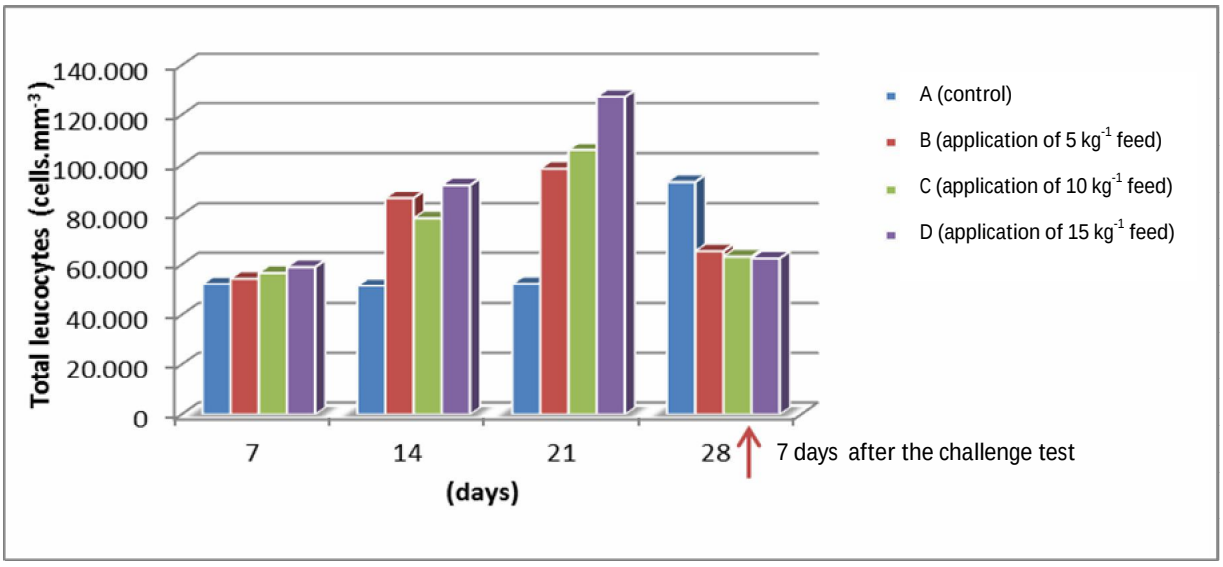

Figure 2. Total leucocyte of *O. niloticus* during saluang belum application and after the challenge test.

Total leucocytes of Nile tilapia during the study were in the normal range for all treatments, between 20,000 and 150,000 cells mm^{-3} (Putra et al 2015). Each treatment yielded different development of total leucocytes for 21-day saluang belum extract application (Figure 2). Leucocyte functions in fish defense system against phatogen (Anderson & Siwicki 1995). When infection occurs, leucocytes are sent to the locus to give quick defense against the gene infection (Sadikin 2002).

Increased total leucocytes in fish administered with immunostimulant indicates that the immunostimulant entering the fish body has given positive effect on total leucocyte development in the blood. It could result from antagonic response of the fish body against the phatogen in the form of increased phagocytic cell activity that functions to demolish alien materials entering the fish body (Nurjannah et al 2013). Phagocytosis is an early stage of body defense mechanisms (Harikrishnan et al 2011). Increased leucocyte population is caused by increased cell division activity and immunostimulant used is mitogenic. Mitogenic compound will activate defense cells to differentiate, result in DNA synthesis in lymphocyte cells, and eventually increase the leucocyte (Faulmann et al 1983; Bly et al 1986; Rorstad et al 1993). Increase in number of total leucocytes reveals humoral and cellular response of leucocytes to overcome the presence of the bacteria (Erlinger et al 2004; Finlay & McFadden 2006; Magnadottir 2006).

Various secondary metabolites of plants possess antibacterial activity through a variety of synergic working mechanisms. The efficacy of herbal extract used in medication could result from the synergy between active compounds in the extract. Synergy gives better activity and reduces potential toxicity of several single compounds and can prevent drug resistance. The synergy of various secondary metabolites is also claimed to be able to reduce undesired side effect (Poongothai & Rajan 2013; Hernani 2011).

The working mechanism of tannin as antibacterial is to inhibit reverse transcriptase enzyme and DNA topoisomerase so that the bacterial cells cannot be formed (Nuria et al 2009). Tannin has antibacterial activity in relation with its ability to activate the adhesine of microbial cells, activate enzyme, and disturb protein transport in the cell internal layer (Cowan 1999). Tannin also has target on the polypeptide of the cell wall so that the cell wall formation is not perfect. Tannin causes the bacterial cells become lysed due to osmotic or physical pressures so that the bacterial cells die (Sari & Sari 2011). The complexation of iron ion with tannin could explain tannin toxicity. The

microorganisms growing under aerobic condition need iron for various functions, including ribonucleotide DNA reduction and precursor. Reverse transcriptase enzyme and DNA topoisomerase of the bacterial cells cannot be formed by strong iron binding capacity of tannin (Akiyama et al 2001).

The working mechanism of saponin as antibacterial is to make protein and enzyme leakage from internal cell (Madduluri et al 2013). Saponin can become antibacterial since the surface of the active compound is like detergent, so that it will inhibit the fitness of the cell wall permeability of the bacteria. This damaged cell membrane highy disturbs the survival of the bacteria (Harborne 2006). Saponin diffuses through the vulnerable external membrane and cell membrane, then binds the cytoplasmic membrane that disturbs and reduces the cell membrane stability. It makes the cytoplasm leak out from the cell and causes cell mortality (Cavalieri et al 2005).

Figure 2 shows that 7 days after the challenge test, treatments B, C, and D have total leucocyte reduction. It could result from increased cortisol level as the effort of the infected fish to recover from stress condition after bacterial infection. Treatment A had increased leucocytes after infection indicating the presence of antigen (*A. hydrophila*) entering the fish body through injection. Increased number of leucocyte cells could result from increased cellular defense against the bacterial infection that triggers the cell division activity (Hardi et al 2011; Matofani et al 2013).

Survival rate (SR). The use of saluang belum extract gave different effect with treatment. The lowest SR occurred in the control treatment (A), 46.67% and the highest at the treatment dose of 5 g kg^{-1} feed (D), 89.00%. Low survival rate in the control treatment (A) indicates that the innate fish immune is low.

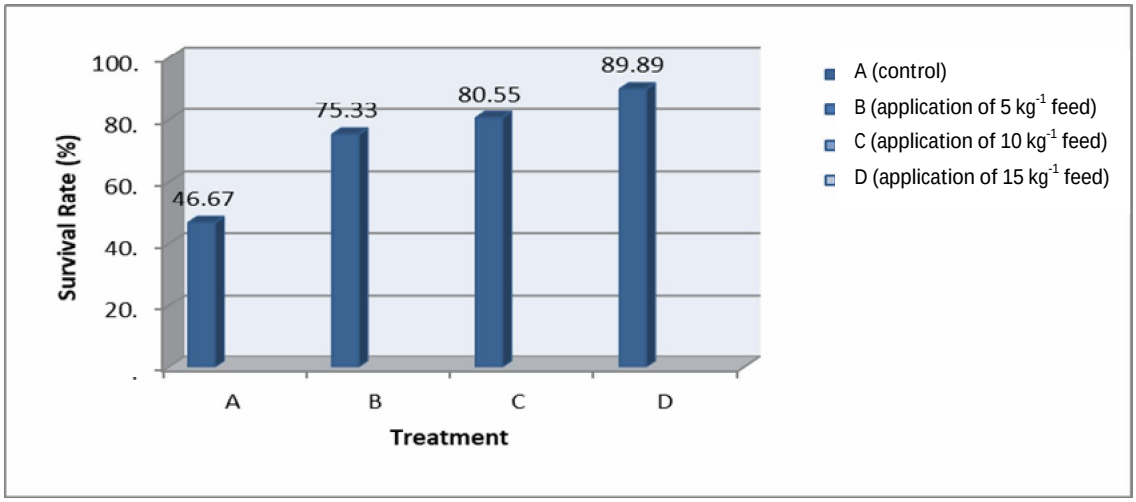

Figure 3. Survival rate (SR) of *O. niloticus* during the study.

The present study revealed that salang belum extract could inhibit the growth of pathogenic bacteria *A. hydrophyla* indicated with high SR of Nile tilapia compared with the control treatment. It is related with the ability of saluang belum to have pharmacological activity as immmunomodulator. The secondary metabolites in saluang belum extract are tannin and saponin that are potential to inhibit the bacterial growth (Okoli et al 2009; Suhirman et al 2010). Tannin activity in bacterial growth inhibition is related with its ability to bind the cell membrane of the bacteria and inhibit the growth and the protease activity (Jones et al 1994). The activity of saponin compound in bacterial growth inhibition is to reduce the efficiency of glucose utilization in microorganism, influence growth and proliferation, decrease the key enzymatic activity in physiological metabolism, and suppress protein synthesis, then kill the bacteria (Yu et al 2013; Akinpelu et al 2014).

Water quality. During the study, water quality of the culture tank was measured covering temperature, pH, dissolved oxygen, and ammonia. Results revealed that water

quality of culture tank was in the tolarable range for the fish culture. Water temperature was in the range of 25-29ºC, pH 6.1-7.3, DO 4.2-7 mg L^{-1}, and NH_3 0.072-0.528 mg L^{-1}. It is supported by Nisa et al (2015) that ideal pH for aquatic organisms, in general, ranged from 7 to 8.5, whereas good pH for freshwater fish culture ranges from 6 to 9. Good water quality parameters for growth and development of Nile tilapia is 3 - >5 mg L^{-1} for dissolved oxygen (Arifin 2016), 25-30^{o}C for water temperature (Kordi 2009), and less than 1 mg L^{-1} for ammonia (Tatangindatu et al 2013).

Conclusions. Application of saluang belum extract could increase the immune response, such as phagocytotic activity, total leucocytes, survival rate, of Nile tilapia. The best dose that could give immune response was 15 g kg^{-1} feed. This dose made the phagocytotic activity in the fish administered with saluang belum extract. The highest value was recorded at the dose of 15 g kg^{-1} feed, 34.12%, quite different from the control treatment (without addition of saluang belum immounostimulant), 17.51%. Total leucocytes were also the highest at the dose of 15 g kg^{-1} feed, 127,400 cells mm^{-3}, where the control treatment had only 52,370 cells mm^{-3}, and in day-28 or 7 days after the challenge test, total leucocytes were lower at the dose of 15 g kg^{-1} feed. The highest SR was found at the dose of 15 g kg^{-1} feed, 89.00%, whereas the control treatment had only 46.67%.

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Received: 27 January 2021. Accepted: 18 February 2021. Published online: 28 February 2021.
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How to cite this article:
Rozik M., Maryani, Rosdiana, Rochman A., 2021 Non-specific immune response development of *Aeromonas hydrophila*-infected Nile tilapia *Oreochromis niloticus* with application of immunostimulant of saluang belum (*Luvunga sarmentosa*) extract. AACL Bioflux 14(1):536-545.