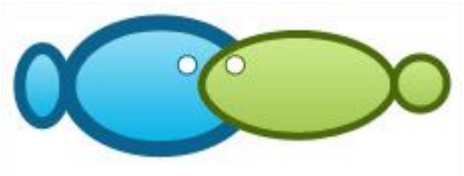

Effectivity of *Arcangelisia flava* as immunostimulant to prevent streptococcosis on Nile tilapia, *Oreochromis niloticus*

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Abstract. This study aims to estimate the effectivity of *Arcangelisia flava* Merr. application in feed as immunostimulant on total leucocyte, neutrofil, monocyte, lymphocyte, and survivorship of Nile tilapia *Oreochromis niloticus* infected with *Streptococcus agalactiae*. Data were descriptively presented and qualitatively described in the form of histograms. Results showed that the highest number of leukocytes was found in treatment E, 81.534 cells mL^{-1} and the lowest in treatment A, 21.379 cells mL^{-1}. The highest number of neutrophils occurred in treatment E, 35.25% and the lowest in treatment A, 30.26%. The highest number of lymphocytes was recorded in treatment E, 80.50% and the lowest in treatment A, 66.50%. The highest number of monocytes count also occurred in treatment E, 13.00%, and the lowest in treatment A, 9.00%. *A. flava* could also affect the survivorship. The highest survival was found in treatment E, 91.66%, and the lowest in treatment A, 76.33%. This finding indicated an increase in total leucocyte, neutrophil, monocyte, and lymphocyte, and the survival rate of *O. niloticus* infected by *S. agalactiae* at treatment of 200 ppm compared with the control treatment. It reflects that 200 ppm of *A. flava* is the most effective concentration, in which this dose is immunogenic and protective in raising the immunity of *O. niloticus* against *S. agalactiae* infection.

Key Words: immunostimulant, leucocyte, neutrophil, monocyte, lymphocyte.

Introduction. Disease infection is a constraint in aquaculture that can reduce the fisheries productivity. According to Park et al (2009), streptococcosis is caused by bacteria *Streptococcus* sp. and one of the diseases that often attack Nile tilapia, *Oreochromis niloticus* and can increase fish mortality up to 50%. The disease comes up when significant temperature fluctuation occurs, so that the immune system of the fish decreases. *Streptococcus* sp. enters the body through digestive system (Irianto 2004). The initial symptoms exhibit that the fish stomach looks bigger. The bacteria will enter the blood vessels reaching the kidney. The fish kidney infected with *Streptococcus agalactiae*, particularly Nile tilapia, will look pale and swell. The fish will show whirling movements when the bacteria *Streptococcus agalactiae* infects the brain's nerve system. The disease symptom can also be known from swelling and bleeding in the fish eyes (exophtalmia) (Filho et al 2009).

Prevention of streptococcocis is still done using antibiotics. However, the effect of continuous use of the antibiotics can yield the resistance of pathogenic microorganisms to the antibiotic and leave some residues in fish and their environment (FAO 2008; Widya et al 2014). Therefore, safer mitigation and disease prevention alternatives are needed for both fish and their environment.

The use of immunostimulant can be conducted as protection and prevention efforts of disease infections in fish. It can increase the natural and adaptive immune system of the fish (Kani et al 2003). Immunostimulant has higher superiority than other methods, since it is capable of increasing the non-specific immune response of the

aquatic animals to prevent various disease infections from bacteria or viruses (Sakai 1999; Galindo-Villegas & Hosokawa 2004; Barman et al 2013; Labh & Shakya 2014).

Arcangelisia flava has been known by Central Kalimantan communities, especially Dayak people as yellow root and utilized as traditional medicine with its ability to cure various diseases, one of which is hepatitis. Phytochemical test revealed that *A. flava* was detected to hold secondary metabolites, namely alkaloid, saponin, terpenoid, and flavonoid (Maryani et al 2013). All active compounds contained in *A. flava* enable it to be developed as an imunostimulant.

The objective of the study is to know the effectivity of *A. flava* in the feed as immunostimulant on total leukocytes, neutrophils, monocytes, and lymphocytes, and the survivorship of Nile tilapia infected with *Streptococcus agalactiae*.

Material and Method. This study was carried out from May 10th to June 11th, 2017. Test animals were Nile tilapia taken from Freshwater Aquaculture Development Center in Mandiangin, Banjar Regency, South Kalimantan, with mean size of 8.0±1.2 cm, as many as 20 ind tank^{-1}. Each treatment had 3 replications, so that there were 360 individuals of fish employed. The fish were firstly acclimated in the fiberglass tank for 7 days to guarantee no streptococcocis symptom and other infection. During acclimatization, the fish were fed *ad libitum* twice a day and siphoned every morning before feeding.

A. flava extract was obtained through extraction, as much as 2 kg stem of *A. flava* was wind-dried. The dry material was blended and then macerated in 8.5 L of 96% ethanol for 24 hours until the supernatant was formed. The supernatant was evaporated using vacuum rotavapour (N-1001S-W, EYELA-USA) at 40^{o}C at a speed of 120 times per minute. The extract was diluted using distilled water with treatment concentration.

Bacterium *S. agalactiae* used in the test was obtained from Freshwater Aquaculture Development Center of Mandiangin, Banjar Regency, South Kalimantan. Before use, the bacterium was isolated on Brain Heart Infusion Agar (BHIA) sterilized in the autoclave. The isolate of *S. agalactiae* was taken using ose needle and inserted into the agar media using scratch method and incubated at 37^{o}C for 24 hours.

The commercial feed used contained 30-32% protein. It was added with *A. flava* extract with treatment concentrations 0 ppm (A), 50 ppm (B), 100 ppm (C), 150 ppm (D), 200 ppm (E), and 250 ppm (F). Extract addition was done by evenly spraying the feed up to be homogenously mixed and then wind-dried. *A. flava* extract-containing feed was given at 5% of body weight twice a day for 21 days and then exposed to infection of bacterium *S. agalactiae* in the water at the doses of 4 mL with a density of 10^7 cfu mL^{-1} for each aquarium.

This study was experimental using Complete Randomized Design with 6 treatments and 3 replications. Parameters observed were total leukocytes, neutrophils, monocytes, and lymphocytes, and the survivorship. The survival rate of Nile tilapia was assessed using Effendie (1979):

$$SR = Nt/No \times 100$$

where: SR = survival rate;
Nt = number of fish at the end of observation;
No = number of fish at initial study.

Supporting parameters measured were water temperature, pH, and dissolved oxygen (DO). Measurements of water temperature, pH, and DO were done every morning to control the culture environmental conditions.

Results and Discussion

Leukocytes. Number of leukocytes at treatment A, B, C, D, and E increases, but it decreases at treatment F (Figure 1). The highest number of leukocytes after treated with *A. flava* extract was 81.534 cells mL^{-1} at treatment E and the lowest was 21.379 cells mL^{-1} at treatment A, indicating that treatment E is an appropriate dose of the

immunostimulant to support the immune system well. Increased mean total leukocytes could result from the effect of *A. flava* extract administration.

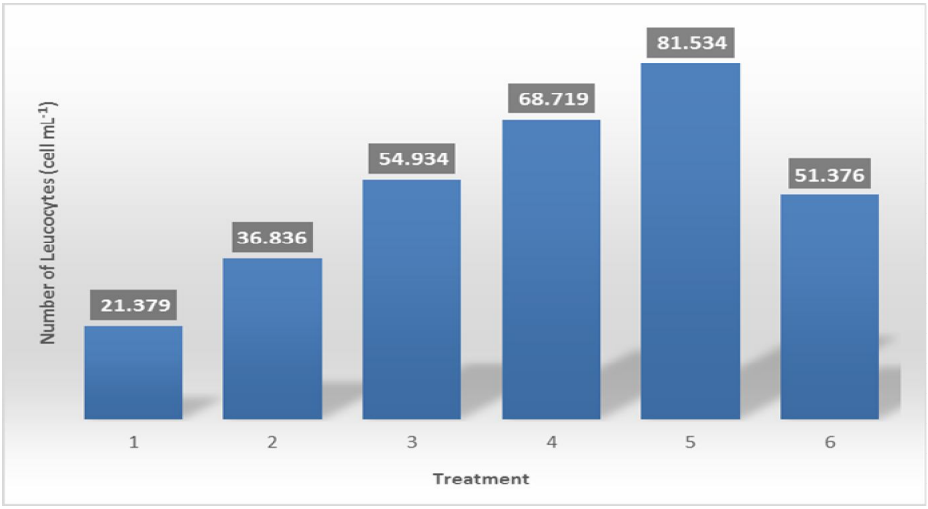

Figure 1. Number of leukocytes in Nile tilapia treated with *A. flava* under different doses after infected with *S. agalactiae*.

Imunostimulant is a chemical compound of drugs or other materials that is capable of increasing the specific and non-specific response mechanisms of the fish (Anderson 1992; Galeotti 1998; Mastan 2015). The administration of *A. flava* extract as much as 50 ppm to 250 ppm to the test fish exhibited increment of total leukocytes and gave significantly different effect from that of 0 ppm. An appropriate dose of immunostimulant administration will raise the complementary activities, opsonin macrophage, phagocytosis, and polymorphonuclear leukocytes (PMN) that help eliminating the entering anti-gene so that fatal infection development does not occur. Increased number of total leukocytes indicates the humoral and cellular leukocyte responses to prevent the presence of bacteria (Erlinger et al 2004; Finlay & McFadden 2006; Magnadottir 2006).

Leukocytes play important role in fish defensive system against pathogenic infections (Anderson & Siwicki 1995). When infections occur, the leukocytes will be transported to the infected past to give quick protection against the infectious gene (Sadikin 2002). According to Fujaya (2004), leucocyte is one of the blood groups possessing very crucial role in immune response system of the fish, and it will drastically increase when infection occurs.

The present study revealed that total leukocytes rose with addition of *A. flava* extract dose, except slight decline at the dose of 250 ppm (F), but the number of leukocytes was higher than that in 0 ppm (A). It means that *A. flava* extract holds immunostimulant-containing compounds that could induce the body resistance against *S. agalactiae*. According to Maryani et al (2013), *A. flava* contains secondary metabolites, one of which is flavonoid. The working mechanism of flavonoid is to inhibit the function of cell membrane through complex compound formation against the extracellular protein that disturbs the wholeness of the bacteria cell membrane. It is done by denaturing the bacteria cell protein and by making cell membrane irreparable (Nuria et al 2009; Naiborhu 2002; Sen et al 2015). Other study found that flavonoid mechanism in cell membrane function prevention is to distort the cell membrane permeability, restrain the cell membrane function through cell membrane permeability distortion, and impede the enzymatic binding, such as ATPase and phospholipase (Li et al 2003).

Neutrophils. Figure 2 shows that number of neutrophils increases at treatment B, C, D and E, but declines at treatment F. The highest increment was recorded at treatment E, 35.25 %, and the lowest at treatment A, 30.25%.

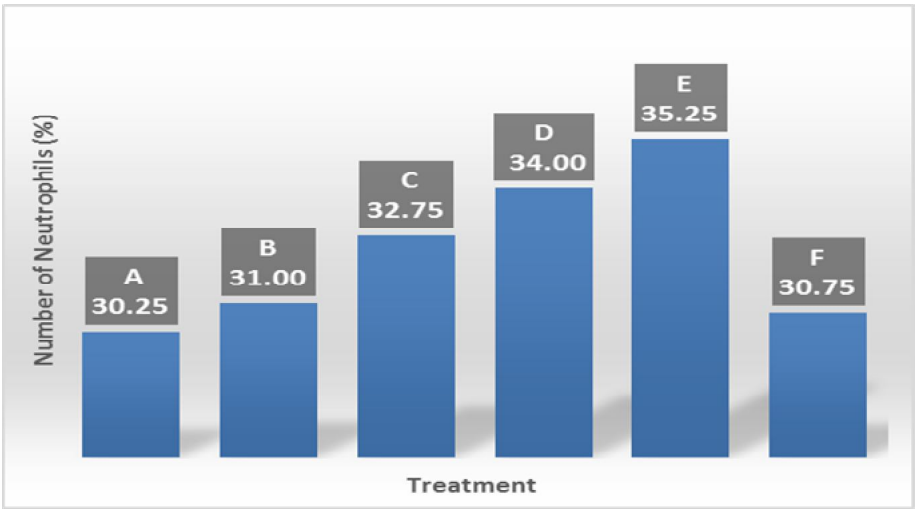

Figure 2. Number of neutrophils in Nile tilapia treated with *A. flava* under different doses after infected with *S. agalactiae*.

Neutrophils also rose with increased dose of *A. flava* extract and slightly declined at the treatment of 250 ppm (F), but they were higher than those at control treatment. Neutrophils work to conduct phagocytotic activities against antigens entering the fish body. This study found that increased total leukocytes were followed with increased number of neutrophils. In general, number of neutrophils increases at bacterial infections since they are released from the blood vessel to the infected location. Major function of neutrophils is to destroy alien materials through phagocytosis, i.e. chemotaxis, particle attachment to the cell, particle ingestion by the cell, and particle destruction by lysosome in the phagosome (Tizard 1998; Karuthapandi & Innocent 2010). Releasing neutrophils from the blood vessels when infection occurs could be caused by external chemical stimulation or chemotaxis (Vadstein 1997; Kozinska & Guz 2004; Misra et al 2006).

Neutrophil is polymorphonuclear (PMN) cell with the shortest life time and will die through apoptosis, approximately 24 hours. Increased number of neutrophils could result from the entry of alien matter in the fish body. This increase could result from immunostimulant administration of *A. flava* extract containing secondary metabolites, one of which is flavonoid. This compound has direct immune effect and stimulates the granulocytes development indicated with increased amount of neutrophils in the fish body (Lou et al 2011). Neutrophils can yield monocyte and macrophage stimulating substance. The secretion can raise the phagocytosis and ROS involved in intracellular killing. This cell is a powerful phagocyte. Phagocytosis is done by approaching the foreign particle and releasing pseudopodia to all directions around the particle. A neutrophil can phagocytize 5-20 bacteria (Tizard 1998).

Lymphocytes. Treatments A, B, C, D, and F resulted in lymphocyte range from 66.50% to 78.25%. The range reflects that the lymphocytes are in normal condition (Galindo-Villegas & Hosokawa 2004), 60-85%, but lower than that of normal fish reported by Smith (2007), > 80%. In treatment E, number of lymphocytes increased up to 80.50%. It means that humoral immune response of the fish is in good condition, so that the resistance to the alien material and antibody formation occur. Increased number of lymphocytes occurs as the lymphocytes that are temporarily in the blood will migrate to various lymph nodes or into the spleen. When they meet the alien materials, the lymphocyte will grow and undergo mitosis of plasma cell that functions as antibody producer (Mishra et al 2009).

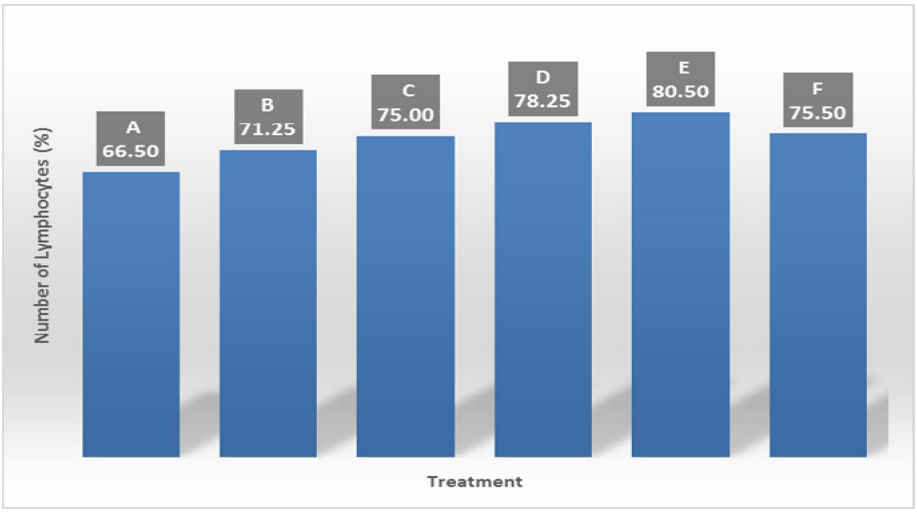

Figure 3. Number of lymphocytes in Nile tilapia treated with *A. flava* under different doses after infected with *S. agalactiae*.

This result is supported by Mundriyanto et al (2002) that the role of lymphocytes in the immune system is to release immune substances for body protection by detecting the antigens through specific receptor of the cell membrane. The mechanism of the lymphocyte number development is as follows: lymphocyte cells occur in the blood and will migrate to the lymph and stay there. When encountering foreign substances, the lymphocyte will develop and undergo mitosis to become plasmoid as antibody producer (Sadikin 2002).

Number of differential leucocytes of each type of blood cells, neutrophils, monocytes, and lymphocytes, will rise in sick fish, since the leucocyte component will fight against the pathogens. It is in agreement with Fujaya (2004) that leucocytes will enter the blood in great number after infection. Leucocytes are believed acting as protein detoxication before causing damages in the organism.

Monocytes. The present study found that the highest fish monocytes were recorded in treatment E, 13.00%. This condition could result from the alteration of monocyte to macrophage and migrate to the infected locations to phagocytize the antigens of *S. agalactiae*.

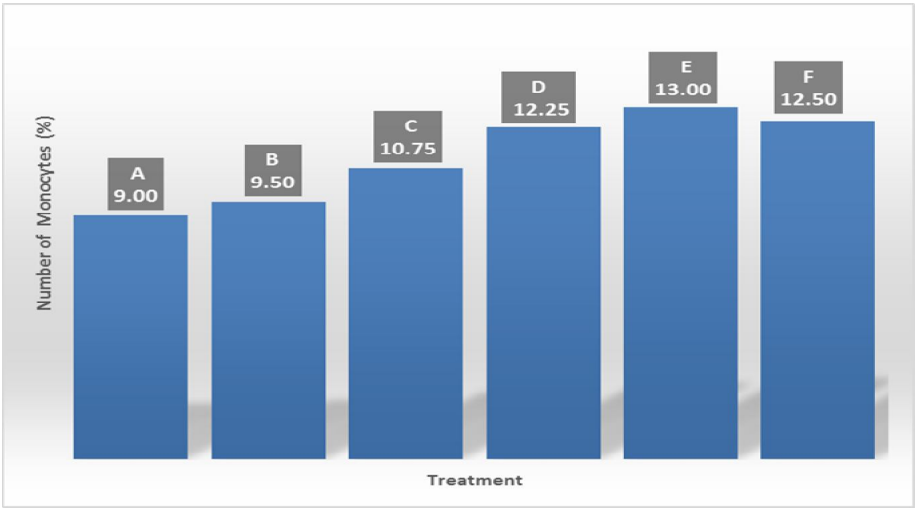

Figure 4. Number of monocytes in Nile tilapia treated with *A. flava* under different doses after infected with *S. agalactiae*.

Increased monocytes during infection, according to Selvaraj et al (2005) stimulate the monocyte to destroy the bacteria. Monocytes are cells in the blood vessel and develop to macrophage when they are activated, in which macrophages possess stronger phagocytic ability than neutrophils even though the granulocytes have higher amount. Maftuch (2007) also describes that inflammation from infection or antigen-antibody reaction will increase monocyte production twice more.

According to Bijanti (2005), monocyte distribution in the blood becomes shorter (1-2 days), and then the cell migrates in the tissues where it differentiates further to be macrophage. Secombes (1990), Anderson & Siwicki (1995), Bastami et al (2009), and Harikrishnan et al (2010) added that proportion of monocytes was low in leukocyte population, but it could rise about 38% in short time when infection occurred.

Survivorship. The present study found that the highest survivorship of fish treated with *A. flava* extract was recorded in treatment E, 91.66%, and the lowest in treatment A, 76.33% (Figure 5). It reveals that treatment E is the most effective compared to other treatments.

High survivorship of the test fish after *A. flava* extract administration reflects the potential of the extract to reduce *S. agalactiae* population. It could be due to the presence of antibacterial substances in *A. flava,* flavonoid. Bacterial infection in fish could occur through wounded body surface, food or gills. Then the bacteria enter the blood vessels, disperse, and yield blood poisoning. *S. agalactiae* can also infect the fish since they can recognize certain cell receptors, then the bacteria kill and break down the host cells by producing the extracellular enzymes, and used the breakdown host cells as nutrients for their growth.

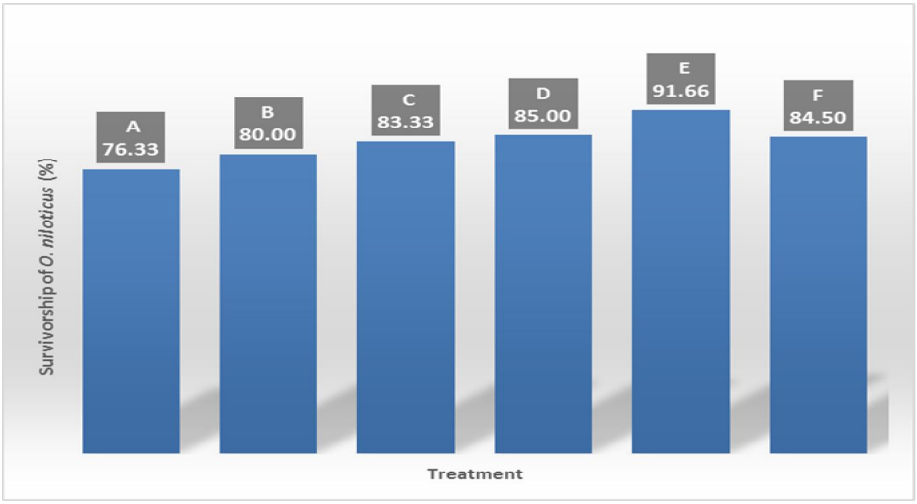

Figure 5. Survivorship of Nile tilapia treated with *A. flava* under different doses after infected with *S. agalactiae*.

The population development of pathogenic bacteria causes inflammation around the infected location and makes the wound get worse to be a haemorrhage. The breakdown of fish body cells in the infected area damages the blood vessel, the pathogenic bacteria enter and disperse through blood circulation to the entire fish body. If these haemorrhages attack the important organs, such as respiration organs, digestive tract, kidney, and liver, they will cause mortality (Jun et al 2010).

The presence of flavonoid in *A. flava* extract as antibacterial is to denature the protein cells, destroy the bacterial cell membrane so that the bacteria cannot grow, and prevent toxin release until the bacteria are killed (Hendra et al 2011; Rijayanti 2014). Besides, flavonoid is an antioxidant as well and capable of increasing the immune system function because the leukocytes as antigens feeder are produced faster and the lymphoid system is also activated faster. The present finding is supported by Susilo (2012) that flavonoid compounds contained in dandelion (*Taraxacum officinale*) extract can raise the

immune activities. According to Suhirman & Winarti (2013), the secondary metabolites of plants, such as flavonoids, are potential as immunostimulant, antibacterial, antiviral or other microorganisms. The potential of flavonoids as immunostimulant has also been reported by Susanti et al (2012) in cinnamon (*Cinnamomum verum*) extract against *Salmonella enteritidis*, by Ulfah (2014) for roselle (*Hibiscus sabdariffa*) flower, and by Kusmardi et al (2007) in tropical shrub (*Cassia alata*) leaf.

Water quality. Poor water quality condition could inhibit growth and cause disease in fish. Water temperature during the study ranged from 27 to 29^{o}C, and it could support the survivorship of Nile tilapia. It is in agreement with Khairuman & Amri (2012) that optimum water temperature for feeding activities ranged from 25 to 27^{o}C. Water pH ranged from 6.90 to 7.30 during the study, highly supported the survivorship of Nile tilapia, and this range belongs to good condition for fish production suggested by Cholik et al (2005). Dissolved oxygen ranged from 5.00 to 6.60 mg L^{-1} and these are higher than that required by National Standard Bureau of Indonesia (BSN 7550: 2009), 3.0 mg L^{-1}, for culture media of Nile tilapia.

Conclusions. Application of *A. flava* as non-specific immunostimulant in the fish feed could raise the amount of leukocytes, neutrophils, lymphocytes, and monocytes in Nile tilapia infected with *S. agalactiae.* The highest number of leukocytes, neutrophils, lymphocytes and monocytes was found in treatment E, 81.534 cells mL^{-1}, 35.25%, 80.50%, and 13.00% respectively. The administration of *A. flava* in the feed as non-specific imunostimulant affected the survival rate of Nile tilapia infected with *S. agalactiae* with the highest in treatment E, 91.66%, and the lowest in treatment A, 76.33%.

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