



Immunostimulant activities of yellow root (*Arcangelisia flava* Merr.) extract on *Edwardsiella tarda* infection

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Abstract. *Edwardsiella tarda* is edwardsiellosis-causing bacterium that infects freshwater and marine cultured fishes. One of the safe alternatives to prevent *E. tarda* infection in fish, human, and environment is nature-derived immunostimulant application, such as typical forest plants from Central Kalimantan, yellow root *Arcangelisia flava* Merr. This study was aimed at examining the immunomodulator activity of yellow root in non-specific resistance of catfish *Pangasionodon hypophthalmus* infected with bacterium *E. tarda*. The fish were treated with yellow root extract soaking at different doses as follows: Kn – without yellow root extract soaking and not infected with bacterium *E. tarda*, Kp – without soaking in yellow root extract and infected with *E. tarda*, P1 – soaking in 0.3 g L⁻¹ of yellow root extract and infected with bacterium *E. tarda*, P2 – soaking in 0.5 g L⁻¹ of yellow root extract and infected with bacterium *E. tarda*, P3 – soaking in 0.7 g L⁻¹ of yellow root extract and infected with bacterium *E. tarda*. Parameters measured were total leucocyte, hematocrit level, phagocytic activities and survival rate. Results indicated that the yellow root extract at the highest doses, 0.7 g L⁻¹, was the most effective dose to be used as immunostimulant in *P. hypophthalmus*. The yellow root extract can raise total leucocyte, hematocrit level, phagocytic activity, and the survival rate that are beneficial for overcoming *E. tarda* infection.

Key Words: catfish, edwardsiellosis, *Edwardsiella tarda*, immunostimulant.

Introduction. *Edwardsiella tarda* causes a disease called edwardsiellosis or Emphysematous putrefactive disease (Susanti et al 2016) that is an important bacterial infection of freshwater and marine fishes. Emphysematous putrefactive disease has considerable economic effects on the aquaculture industry. *E. tarda* is an important zoonotic pathogen as well (Mainous et al 2010). Therefore, accurate prevention efforts need to be done to control the infection. Fish disease prevention in aquaculture is conducted using chemical substances or antibiotics, such as ampicillin, chloramphenicol, and tetracycline. The wide use of antibiotic is related with bacterial resistance to the antibiotics, and can leave the antibiotic residue in the fish body as well that possibly hazards human consumption (Endah et al 2014). Disease prevention has been recently done using natural materials or medicinal plants as source of immunostimulants or antimicrobials. Several benefits of using natural materials are that they are relatively safer, easily obtained, cheap, not causing resistance, and relatively environmental friendly (Edwina et al 2017; Nya et al 2017).

Indonesia's biodiversity has the potential of providing various natural medicines or functional food from terrestrial or aquatic plants. Central Kalimantan forest still holds numerous plants for potential medicine development. Yellow root plant *Arcangelisia flava* is one of the forest plants used as traditional medicines for Central Kalimantan people, particularly Dayakness with hepatitis. This plant is reported containing saponin, flavonoid, terpenoid, and alkaloid as antimicrobial (Maryani et al 2013). The presence of these compounds makes it be efficacious as immunostimulant.

Immunostimulant is simply a substance stimulating or increasing the immune system through direct interaction with cells activating the immune system (Barman et al

2013; Wang et al 2017; Ghasemian et al 2017). The immunostimulant entering the body will stimulate the macrophages to produce interleukin that then activate the lymphocyte cells to become T lymphocytes and B lymphocytes. The former will produce interferon that is capable of reviving the macrophages to phagocytize the bacteria, viruses, and other alien particles entering the body (Secombes & Wang 2012; Nurkhasanah et al 2017). The immunostimulant can be bacteria and bacterial products, yeast, carbohydrate complex, nutritive factors, animal extract, plant extract, and synthetic drugs (Elsayed et al 2015).

This study aims to know the effect of yellow root *A. flava* extract application as non-specific immunostimulant at different doses on total leucocytes, hematocrit level, phagocytic activity, survival rate (SR) of catfish *Pangasionodon hypophthalmus* infected with bacteria *E. tarda*.

Material and Method. This study was accomplished in October 2017 - January 2018 in the Laboratory of Fish Health, Department of Fisheries, Faculty of Agriculture, Palangka Raya University. It employed yellow root extract, 8-12 cm total length-sized catfish *P. hypophthalmus*, bacterial isolate of *E. tarda*, alcohol, tryptic soy agar (TSA), tryptic soy broth (TSB), glutamate starch phenol (GSP), ethylene diamine tetraacetic (EDTA), HCl, phosphate buffer saline (PBS) solution, hayem solution, and commercial pellet. The equipment used were Eppendorf tube (OneMed), micropipette (Scilogex), analytical balance (Ohaus), flask (Pyrex), Petri disc (Pyrex), autoclave (HL-36AE Hirayama), incubator (Memmert), syringe (OneMed), binocular microscope (Olympus), vacuum rotavapour (N-1001S-W, EYELA-USA), haemocytometer (Assistant), object and cover glass (Sail Brand), DO-meter (OM-70 Horiba), pH-meter (Hanna Instruments), and spectrophotometer (Amtast AMV 11).

The study was experimental with one factor Complete Randomized Design at five levels of treatments and 3 replications. The treatment applications were Kn (without immersion in yellow root extract and no *E. tarda* infection), Kp (without immersion in yellow root extract and infected with *E. tarda*), P1 (immersion in 0.3 g L⁻¹ of yellow root extract and infected with *E. tarda*), P2 (immersion in 0.5 g L⁻¹ of yellow root extract and infected with *E. tarda*), and P3 (immersion in 0.7 g L⁻¹ of yellow root extract and infected with *E. tarda*).

Rearing tank and fish preparation. The rearing tanks were 16 L water tanks. These were washed, rinsed, and filled with freshwater. The tanks were then given 25 ppm of potassium permanganate (KMnO₄) and strongly aerated for 24 h so it can be free of pathogens, rinsed and dried for one day (Asniatih et al 2013).

Preparation of *A. flava* extract. As much as 2 kg of yellow root was wind-dried, blended, and put into a macerator containing 2 L of 96% methanol for 24 hours up to forming supernatant. It was then evaporated using vacuum rotavapor at 40°C at 120 x per min. up to obtaining viscous extract. The yellow root extract was then diluted in distilled water at the concentration of 0.3 g L⁻¹ of extract (A), 0.5 g L⁻¹ of extract (B), and 0.7 L⁻¹ of extract (C), respectively.

Culture preparation of bacteria *E. tarda*. Bacterium *E. tarda* was taken from Freshwater Culture Development Center of Mandiangin, Banjar Regency, South Kalimantan. The bacteria, before use, were isolated on Brain Heart Infusion Agar (BHIA) sterilized in an autoclave. The isolate of *E. tarda* culture was then taken using an inoculum needle and inserted in agar media using spread plate method. The bacterial isolate in BHIA was then incubated in an incubator at 37°C for 24 h. Before challenge test, *E. tarda* was increased the virulence through LD₅₀ test that was done through intramuscular injection at the concentrations of 10⁹, 10⁸, 10⁷, 10⁶, 10⁵, and 10⁴ CFU mL⁻¹. LD₅₀ test indicated that the bacterial concentration used to infect the catfish was 10⁷ CFU mL⁻¹ as much as 0.1 mL ind⁻¹.

Experimental fish. Catfish *P. hypophthalmus* of 8-12 cm long were firstly acclimated for 5 days in fiberglass tank, and fed *ad libitum* 3 times a day (morning, day, and

afternoon). The initial weight was recorded using an analytical balance and then put in the experimental tank at a stocking density of 1 ind 2L⁻¹.

Immersion of *P. hypophthalmus* in *A. flava* extract and infected with *E. tarda*.

The fish were soaked in 5 L of water containing different dose of yellow root extract. During this time, the water was aerated, then the fish were returned to the rearing tank. The fish immersion was carried out 4 times, day-7, 14, 21, and 28, for 30 min. In day-30, the challenge test was done against the bacteria *E. tarda* at the density of 10⁷ CFU mL⁻¹ and dose of 0.1 mL ind⁻¹. After infected, the fish were reared up to day-42.

Blood sampling. Blood sampling was conducted twice, at day-28 and day-42 after the fish had been given yellow root extract and infected with *E. tarda*. Before the blood was taken, the fish had been anesthetized using clove oil of 0.05 mL L⁻¹ water. The blood sampling used 1 mL syringe rinsed in 10% EDTA solution, then inserted into the Eppendorf tube and later employed for total erythrocyte, hematocrit, and hemoglobin observations.

Research parameters. Parameters measured in this study included total leucocytes, hematocrit level, phagocytic activities, survival rate (SR), and water quality, such as water temperature, pH, and DO. Water quality parameters evaluations were done every 7 days, while ammonia concentration was measured at the beginning and at the end of rearing.

Data analysis. Data were presented in the form of histograms. For treatment comparison, ANOVA was used, and then continued with Student Newman-Keuls range test. Water quality data were tabulated and descriptively analyzed.

Results and Discussion

Total leucocyte. The present study found that yellow root extract administration gave very significant effect on the bacteria *E. tarda* ($p < 0.01$). The highest dose 0.7 g L⁻¹ showed higher total leucocytes than those in positive control treatment, negative control treatment, 0.5 g L⁻¹, and 0.3 g L⁻¹. It indicates that the higher the concentration of yellow root extract is administered to the catfish, the higher the total leucocytes of the fish will be (Figure 1).

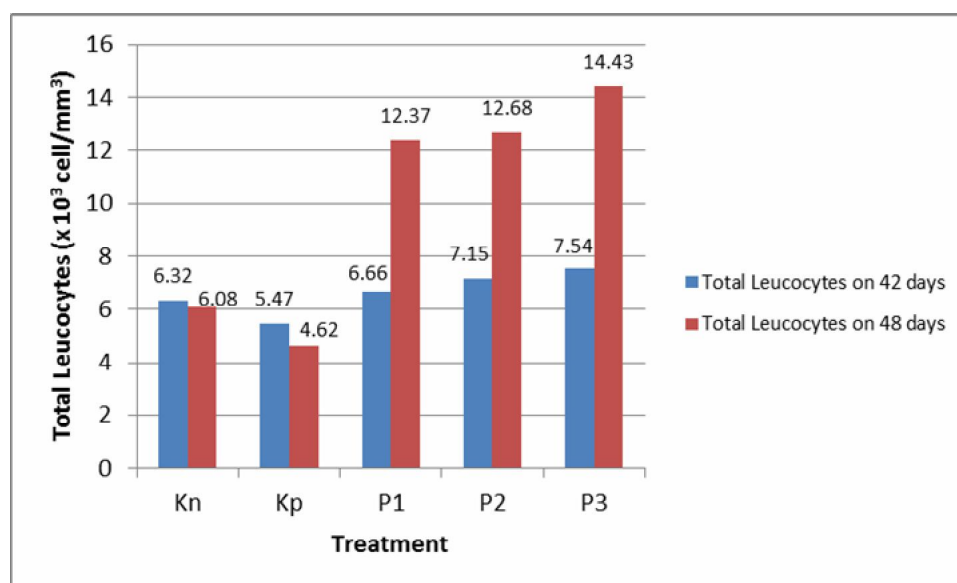


Figure 1. Histogram of total leucocytes in *E. tarda*-infected catfish *P. hypophthalmus* treated with *A. flava* immunostimulant.

The present study showed that yellow root extract could stimulate the development of fish leucocytes. The extract contains compounds that function as immunostimulant to induce the body resistance against bacterial infection of *E. tarda*. According to Maryani et al (2013), yellow root is known holding secondary metabolite compounds, such as alkaloid, saponin, terpenoid, and flavonoid. Steroid, flavonoid, phenol, and tannin that belong to paraimmunity function as mitogen that is able to activate cellular defense (Farina et al 2014). Leucocyte as cellular defense of non-specific defense will phagocytize the pathogens so that the fish body resistance rises due to increased amount of leucocytes (Baleta & Bolaños 2019).

Increase in leucocyte population could result from increased cell division activity. The compounds in the yellow root used as immunostimulant are mitogenic. Mitogen is a substance that induces cell mitosis (Sethi & Singh 2015). The mitogenic compounds will activate the defense cells to differentiate that DNA synthesis occurring in the lymphocyte and increase the leucocyte population (Dewi et al 2014).

Hematocrit level. Hematocrit level estimation was done to know its change after immersion in yellow root extract and infected with *E. tarda*. ANOVA showed that immersion of the catfish in yellow root extract did not give significant effect on the hematocrit level of *P. hypophthalmus* after 28 day-culture or after the challenge test against *E. tarda* ($p > 0.05$). Mean hematocrit level of *P. hypophthalmus* after 28 day-culture with immersion in yellow root extract ranged from 20.35 to 23.54%, while after the challenge test against *E. tarda*, it ranged from 18.73 to 28.22% (Figure 2).

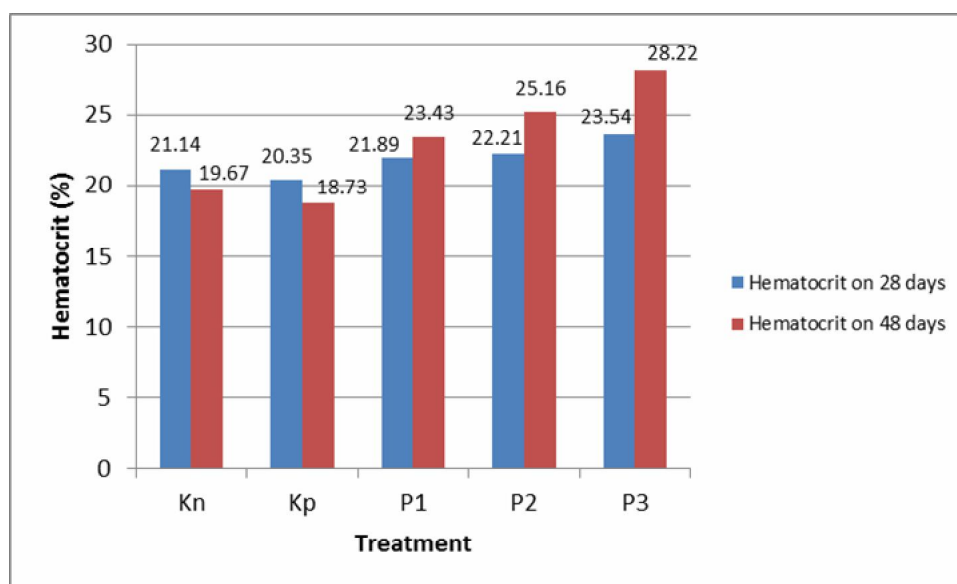


Figure 2. Hematocrit level of *P. hypophthalmus* treated with yellow root immunostimulant.

Based on Figure 2, it is apparent that after *P. hypophthalmus* has been infected with *E. tarda*, the fish hematocrit level rises in all treatments, except in negative and positive controls, 19.67% and 18.73%, respectively. It could result from that at the challenge test, the negative control treatment was not infected with *E. tarda* so that it did not show change in hematocrit level. Moreover, decline in hematocrit level in positive control treatment was due to reduction in number of erythrocytes in the blood and followed with decline in hematocrit level. Ainsyah (2015) stated that there was strong correlation between hematocrit level and number of haemoglobin, in which the lower the number of erythrocytes is, the lower the haemoglobin content in the blood will be.

Hematocrit could be used to know the impact of *E. tarda* infection or as an indicator of fish health after infection. Hematocrit level is also an indication of fish stress condition from environmental factors, handling (injection) or pathogenic infection (Sukenda et al 2014). In this study, the stress-causing factors, such as environment and handling, were minimized, so that increase in hematocrit level could be ensured because

of the pathogenic infection occurrence. Increase in hematocrit levels in treatments P1, P2, and P3 (immersion in yellow root extract) could occur because in post-infection with *E. tarda*, many young erythrocytes (polychromatocytes) were found. In general, young erythrocytes are bigger and more rounded, so that the volume of young erythrocytes will be higher than the normal blood cells (Asniatih et al 2013).

Hematocrit is influenced by several factors, such as erythrocyte condition (amount, size, shape, anticoagulant-blood ratio, storage site, and homogeneity), environment, sex, species, and age, when the fish blood is sampled (Garcia-Roa et al 2017; Septika et al 2017). This finding is in agreement with Septika (2017) that hematocrit level of the catfish *P. hypophthalmus* reared under the administration of feed mixed with God's Crown fruit (*Phaleria macrocarpa (Leucas lavandulifolia)*) solution rises between 24.14 and 26.18% after infected with *A. hydrophila*. According to Sukenda et al (2014), normal hematocrit level of teleostei ranges from 20 to 30%, and fish with anemia have minimum hematocrit of 10%.

Macrophage phagocytotic activity. The macrophage phagocytic activity was estimated to see the macrophagic cell activity development in destroying the antigen entering the body after treated with yellow root extract. This estimation was determined based on the amount of macrophagic cells actively accomplishing the phagocytosis in 100 macrophagic cells (Ainsyah 2015).

Figure 3 demonstrates that the highest mean phagocytic activity occurs in the treatment of 0.7 g L⁻¹ of yellow root extract, 78.37% and the lowest in the positive control treatment, 26.75%. Increased macrophage phagocytic activity with treatments could result from that 0.7 g L⁻¹ extract is the most effective dose to work maximally in raising the macrophage phagocytic activity and potential to be an immunostimulant. Similar finding was also reported by Ainsyah (2015) that the potential of flamboyant leaf extract as immunostimulant can affect the metabolism activity in macrophagic cell. Increased metabolism in the cell will raise enzymes and other substances functioning in the phagocytosis in order to add the phagocytic ability. Increased phagocytic activity after yellow root extract administration could result from the presence of phychemical compound, flavonoid. This compound has been reported by Yuswantina (2012) in the breadfruit *Artocarpus altilis* leaf extract and Susilo (2012) in common dandelion *Taraxacum officinale*, could have immunostimulant effect through the increment of macrophage phagocytic activity. The potential of flavonoid is also found in cinnamon extract against *Salmonella enteritidis* (Susanti et al 2012) and roselle flower *Hibiscus sabdariffa* (Ulfah 2014) in raising the lymphocyte cell proliferation. Arsela & Hesti (2015) who studied corms (*Colocasia esculenta*) found that flavonoid was potential to stimulate the work of lymphokines produced by T cell that the phagocytic cells could be pushed to have the phagocytic response and capable of developing the body immune system activity.

According to Watson & Preedy (2013), lymphocyte activation helper T cells will be activated if there is immune response against the antigen through differentiation to different Th effector cells. These cells will then produce different cytokines depending on the immune response. The Th1 cell will produce interferon gamma (IFN- γ) and interleukin-2 (IL-2) that increase the activity of macrophage cells, natural killer (NK) cells, and cytotoxic T (Tc) cells. This mechanism will quickly increase the phagocytic activity to destroy the antigen and the intracellular microorganisms. Th2 cells will produce several types of cytokines, such as IL-4, IL-5, IL-13, that will stimulate the antibody formation and activate the mast cells and eosinophils in order to raise the defense to the extracellular antigen. This mechanism acts in developing the defense ability against allergic reaction and parasite (Effy et al 2014; Spiering 2015). Nevertheless, flavonoid could directly activate the effector Th1 and Th2 cells to produce cytokine without any immune response to the intracellular or extracellular antigens. The cytokine produced by Th1 and Th2 cells could also enlarge the macrophagic activity (Raphael et al 2015; Azad et al 2018). Thus, flavonoid compounds can improve the phagocytic ability quickly in destroying the antigen and intracellular microorganisms and increase the defense to the extracellular antigens.

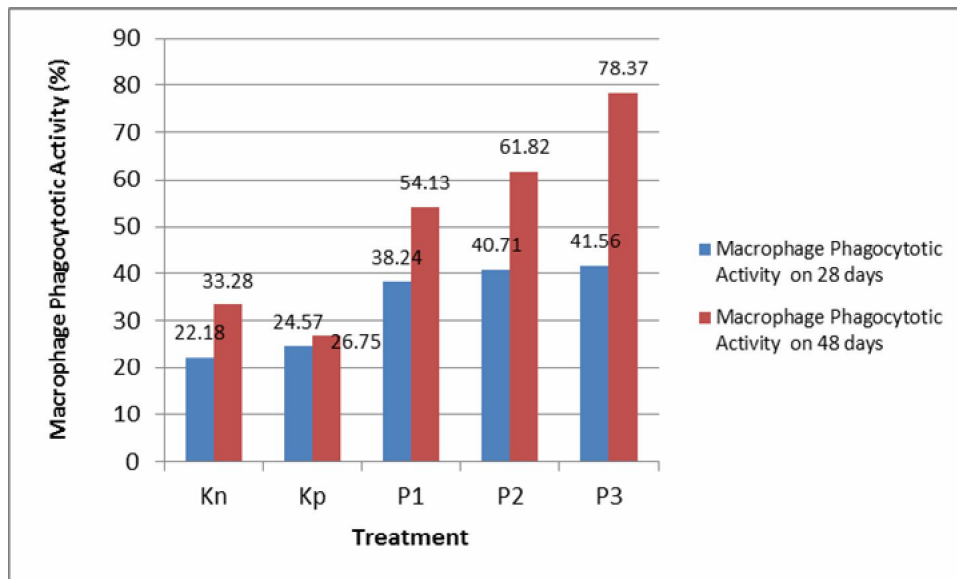


Figure 3. Phagocytotic activity of *E. tarda*-infected *P. hypophthalmus* treated with yellow root immunostimulant.

According to Rosnizar et al (2017), flavonoid content in plants can activate NK cells to stimulate IFN- γ production. IFN- γ produced by various immune cells is major cytokine of Macrophage Activating Cytokine (MAC) that acts in the non-specific cellular immunity (Ivashkiv 2018; Karimi et al 2020). The macrophage can be quickly and efficiently activated by IFN- γ so that the phagocytic activity increases to destroy the antigen.

Survival rate. *P. hypophthalmus* immersed in yellow root extract showed the highest survivorship at the dose of 0.7 g L^{-1} , 93.00% and the lowest in the positive control treatment, 49.67%. Therefore, if compared with the dose treatments of 0.5 g L^{-1} and 0.3 g L^{-1} , the most effective dose of yellow root extract is 0.7 g L^{-1} .

High survivorship of *P. hypophthalmus* after yellow root extract administration could result from the active compound content functioning as antibacterial. According to Suhirman & Winarti (2013), Martins & Nunez (2015), and Soltanian & Fereidouni (2016), the secondary metabolites in plants, such as flavonoid, play a role in raising the body immune system and are able to prevent infections of bacteria, viruses, or other microorganisms. The SR values are presented in Figure 4.

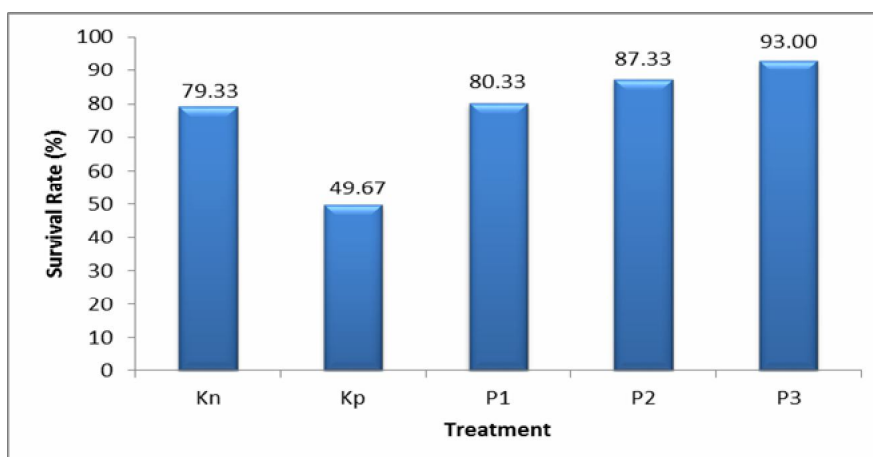


Figure 4. Survival rate (SR) of *E. tarda*-infected *P. hypophthalmus* treated with yellow root immunostimulant.

Water quality. Water quality measurements during the study reflected that the condition was still normal for the fish growth. The water temperature of the rearing tanks

was relatively similar because all water tanks were placed in the same room with the same light penetration. During the study, water temperature ranged from 27.2 to 29.3°C. This range is still secure for fish life and growth as reported by Minggawati (2012) in Irfan et al (2017) that good water temperature for catfish culture ranges 27 to 31°C. Dissolved oxygen ranged from 4.34 to 7.02 ppm since the rearing tanks were aerated during the study. Ammonia concentration was below the quality standard at the beginning of the study, 0.004 ppm, but it exceeded the optimum limit, 0.099 ppm at the end of study. As a whole, water quality conditions could promote good fish growth.

Conclusions. The administration of *A. flava* extract as immunostimulant to the catfish *P. hypophthalmus* increased the non-specific immune system and the survival rate. The best dose treatment to stimulate the non-specific immune system was recorded at the immersion of 0.7 g L⁻¹ *A. flava* extract. This dose made the leucocytes rise to 14.43 x 10³ cells mm⁻³, the hematocrit to 28.22%, mean phagocytic activity to 78.37%, and SR value to 93%.

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Received: 12 March 2020. Accepted: 10 April 2020. Published online: 27 May 2020.

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How to cite this article:

Maryani, Ratnasari I., Alhidayat S. A., Handayani T., Nursiah, Tanduh Y., 2020 Immunostimulant activities of yellow root (*Arcangelisia flava* Merr.) extract on *Edwardsiella tarda* infection. *AAFL Bioflux* 13(3):1336-1344.