

TIK-16 Effects Of Jeruju Leaves Extract (*Acanthus ilicifolius*) On Sangkuriang Cat Fish (*Clarias gariepinus*) Infected With *Aeromonas hydrophila* : Clinical Symptoms and Differential Leukocytes

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EFFECTS OF JERUJU LEAVES EXTRACT (*ACANTHUS ILICIFOLIUS*) ON SANGKURIANG CATFISH (*CLARIAS GARIEPINUS*) INFECTED WITH *AEROMONAS HYDROPHILA*: CLINICAL SYMPTOMS AND DIFFERENTIAL LEUKOCYTES

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ABSTRACT

This study aimed to determine the effect of ethanol extract of *jeruju* leaves on clinical symptoms and differential leukocytes of sangkuriang catfish infected with *Aeromonas hydrophila*. This research was conducted experimentally using a completely randomized design (CRD) consisting of five treatments and three replications. Sangkuriang catfish infected with *A. hydrophila* at a dose of 10^8 cells/mL via intraperitoneal injection. Then, the infected fish were treated with ethanol extract of *jeruju* leaves with concentrations A = 0 mg/L, B = 62.5 mg/L, C = 125 mg/L, D = 250 mg/L and K = control. Parameters observed were clinical symptoms and differential changes in leukocytes, including neutrophils, monocytes, lymphocytes, basophils, and eosinophils. The clinical symptoms of fish infected with *A. hydrophila* after being given *jeruju* leaves extract (according to the treatment concentration) recovered faster than those without the extract (K). The differential leukocytes showed the highest percentage of neutrophils and monocytes at 250 mg/L, basophils at 62.5 mg/L, the highest eosinophils at 125 mg/L, and the percentage of lymphocytes did not differ between treatments. It can conclude that the extract concentrations of 125 and 250 mg/L were effective in stimulating differential leukocytes of sangkuriang catfish, which was indicated by an increase in the percentage of neutrophils and monocytes.

KEY WORDS

Sangkuriang catfish, hematology, differential leukocytes, *jeruju*, *A. Hydrophila*.

Disease outbreaks that attack farmed fish can cause huge losses. Outbreaks that often attack freshwater fish farming, including catfish, are *Aeromonas hydrophila* bacteria. *Aeromonas hydrophila* is an opportunistic pathogen that causes Motile Aeromonad Septicemia (MAS). MAS outbreaks can result in mass death in aquaculture. MAS disease generally attacks freshwater fish such as African catfish (Nitimulyo & Triyanto, 1996), gourami (Mulia, 2005), goldfish (Maji *et al.*, 2006), jambal siam (Olga *et al.*, 2006), giant prawns (Abdolnabi *et al.*, 2015), catfish (*Pangasius hypophthalmus*) (Olga *et al.*, 2020).

In controlling MAS disease, the use of antibiotics in intensive cultivation systems has been prohibited. The prohibition of antibiotics is due to negative impacts such as immunosuppression and accumulation of residues in fish meat tissue and leads to pathogenic bacterial resistance (Harikrishnan *et al.*, 2011). One of the safe ways to control motile aeromonad septicemia (MAS) disease in sangkuriang catfish aquaculture is by utilizing bioactive ingredients from natural products such as *jeruju* plant extract (*Acanthus ilicifolius*). The ethanol extract of *jeruju* leaves has been shown to have potential as an antibacterial of *A. hydrophila* *in vitro* and *in silico* by inhibiting the formation of the peptidoglycan membrane (Aisiah & Rini, 2020). This study aimed to determine the effect of using ethanol extract of *jeruju* leaves (*Acanthus ilicifolius*) on clinical symptoms and differential leukocyte conditions in sangkuriang catfish infected with *A. hydrophila*.



MATERIALS AND METHODS OF RESEARCH

The study was conducted from June to September 2021. The collection of *jeruju* leaves from the riverbanks of Kurau Village, Bati-Bati District, Tanah Laut, South Kalimantan, Indonesia. Then it is taken to the Fish Nutrition Laboratory and the Integrated Laboratory for extraction. Bacteria culture, clinical symptoms, and differential leukocytes were observed at the Laboratory of Fish Pests and Diseases, Faculty of Fisheries and Marine, Lambung Mangkurat University.

The *jeruju* leaves were cleaned, cut into small pieces, and dried at 50 °C until the leaves became dry. The dried leaves were crushed and extracted using a modified method based on Fatin *et al.* (2012) and Aisiah *et al.* (2020). 500 g of *jeruju* leaves powder maceration in 2000 mL of ethanol (Merck) for 4 x 24 hours. The mixture was then separated using Whatman 42 filter paper. The extracts obtained evaporated using a rotary vacuum evaporator at a temperature of 40 °C. Then, the extracts were stored in the refrigerator until they were ready for in vivo testing on infected sangkuriang catfish.

The challenge test was carried out using a completely randomized design. The concentration of *jeruju* leaves extract was determined based on the in vitro inhibition tests, namely the MIC, MBC, and toxicity tests on catfish (Aisiah & Rini, 2020). Sangkuriang catfish infected with *A. hydrophila* at a density of 10⁸ cells/mL. Then soaked with *jeruju* leaves extract for 24 hours, as follows:

- A = Without soaking *jeruju* leaf extracts - 0 mg/L;
- B = Soaking with *jeruju* leaf extracts at 62.5 mg/L;
- C = Soaking with *jeruju* leaf extracts at 125 mg/L;
- D = Soaking with *jeruju* leaf extracts at 250 mg/L;
- K = Control without infected with *A. hydrophila* and soaking with *jeruju* leaf extracts.

The observations were done against clinical and differential leukocyte symptoms, including neutrophils, monocytes, lymphocytes, basophils, and eosinophils from sangkuriang catfish.

RESULTS OF STUDY

Clinical symptoms of sangkuriang catfish for seven days, after the challenge assay (infected) of *A. hydrophila* and soaked in ethanol extract of *jeruju* leaves for 24 hours, are presented in Table 1.

Table 1 – Clinical symptoms of sangkuriang catfish after challenge assay (infected with *A. hydrophila*) and soaked in *jeruju* leaves extract

Observation Time	Clinical Symptoms	
	Infected Fish, with Extract	Infected Fish, without Extract
12 hours	Stretched fish fins, a balance was disturbed, swimming was not normal, slow, eating response was reduced.	Stretched fish fins, a balance was disturbed, swimming was not normal, slow, eating response was reduced.
24 hours	No feeding response and passive movement.	No feeding response and passive movement
36 hours	There was no feeding response, passive movement, some fish were inflamed in the area around the injection, and the fins are slightly damaged.	There was no feeding response, passive movement, some fish were inflamed in the area around the injection, and the fins are slightly damaged.
2 days	some fish were still inflamed, the damage to the fins was getting more visible, some fish had swollen organs, inactive movements, and the response to eating was already there.	Fish were still inflamed, damage to the fins, some fish had swollen organs, inactive movements, and poor response to food
3 days	The fish still had inflammation, damage to the fins, swelling of the organs, but some fish had started to be active and eat.	Fish still had inflammation, swelling; significantly reduced the response to eating.
4 days	Inflammation began to subside; swelling heals, fish began to swim normally and actively move.	Fish are hemorrhagic, swollen, unresponsive to feed, and inactive.
5 days	Inflammation was getting smaller; fish was swimming normally with active movements, response to feed was good.	Fish movement was slow and still hemorrhagic
6 days	No more inflammation, active swimming fish, and good response to feed.	Inactive movement, poor response to eating, fish were still hemorrhagic.
7 days	No more inflammation, active swimming fish, and good response to feed.	Inactive movement, poor response to eating, fish were still hemorrhagic.



The differential observation of leukocytes helps know the difference in the percentage of leukocyte cell components, including the number of neutrophils, monocytes, lymphocytes, basophils, and eosinophils. The percentage of neutrophils, monocytes, lymphocytes, basophils, and eosinophils found in the blood smear of sangkuriang catfish infected with *A. hydrophila* after 24 hours of soaking with *jeruju* leaves extract is presented in Figure 1.

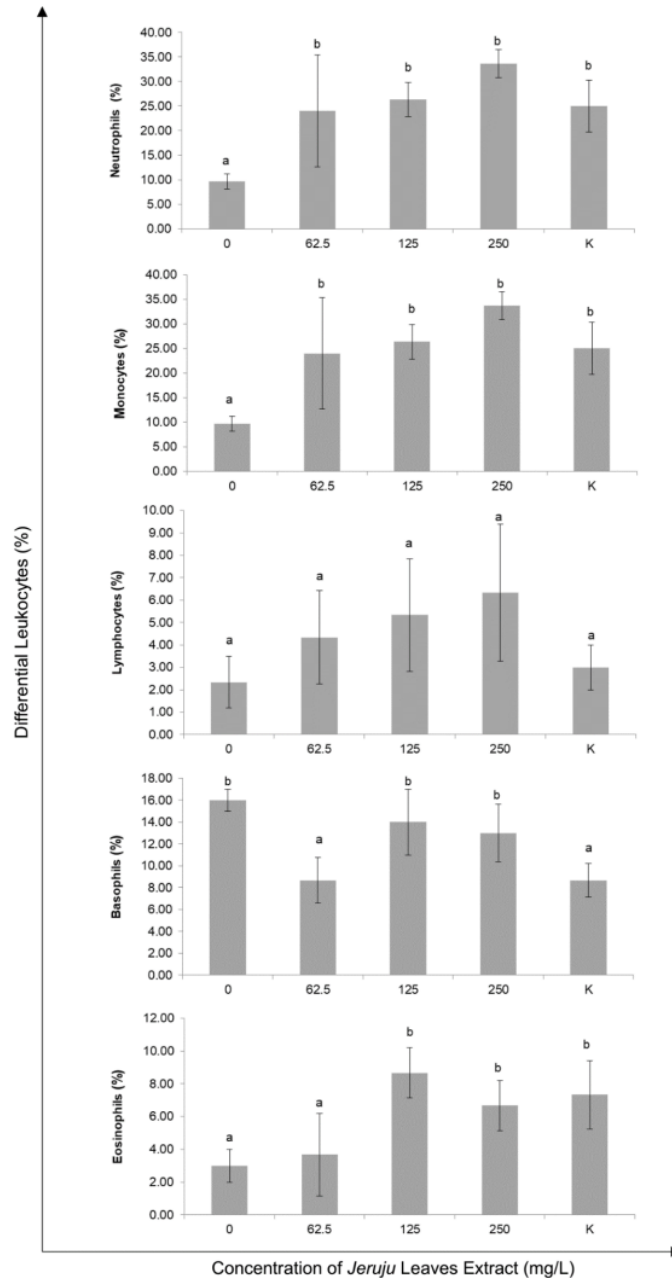


Figure 1 – Differential Leukocytes in Sangkuriang Catfish Infected with *A. hydrophila* and Soaking with *Jeruju* Leaves Extract for 24 Hours



DISCUSSION OF RESULTS

The clinical symptoms shown by sangkuriang catfish after being infected with *A. hydrophila* were inflammation in the area around the injection, wounds that spread around the injection area, damaged fins, swelling (dropsy) occurred, reduced feeding response, and passive movement. Dana (2002) reported clinical symptoms of infection in fish, including empty intestines and did not form a long series, the appearance of changes in body color to become lighter, changes in swimming behavior, reddish tail, and damage to the caudal fin. Motile Aeromonad Septicemia caused by *A. hydrophila* causes various pathological conditions that include acute, chronic, and disguised infections. The severity of the disease is influenced by some interrelated factors, including the virulence of the bacteria, the type and level of stress exerted on the fish population, the physiological conditions of the host, and the level of genetic resistance inherent in a particular population of fish (Cipriano, 2001).

Leukocytes are the most active units of the body's defense system and circulate in the blood circulation in various types. The number of leukocytes is less than the red blood cells. The primary function of leukocytes is to destroy infectious and toxic materials through phagocytosis by forming antibodies. The types of leukocytes observed in this study included the percentage of neutrophils, monocytes, lymphocytes, basophils, and eosinophils. Harikrishnan *et al.* (2003) stated that leukocytes could protect infection from microbes and other chemical factors. Observation of leukocytes can generally explain the immune system and fish health status.

The percentage of neutrophils in the 250 mg/L extracts increased in tandem with the concentrations of 125 mg/L and 62.5 mg/L and was almost the same as in the treatment of healthy fish (K) and decreased in infected fish (0 mg/L). The results of Duncan's Multiple Range Test (DMRT) showed that the percentage of healthy fish neutrophils (K) was not different from the treatment with *jeruju* extract at all concentrations and significantly different from the treatment of infected sangkuriang catfish (0 mg/L). These results indicated that the extract administration affected and increased the number of neutrophils in infected fish.

The neutrophil percentage increased at 250 mg/L, indicating the presence of phagocytic antimicrobial activity. Alamanda *et al.* (2007) stated that neutrophils play a role in the immune response to attacks by pathogenic organisms and have phagocytic properties. Neutrophils in the blood increase in the event of infection and act as the body's first line of defense. Next, Hrubec *et al.* (2000) stated that neutrophils play defense against bacterial infections. Neutrophils normally reside in lymphoid tissue and migrate to blood vessels when inflammatory stimuli occur. At the same time, Suhermanto *et al.* (2011) stated that the primary function of neutrophils is the destruction of foreign materials through phagocytic processes, namely chemotaxis by way of cells migrating towards particles or particle adhesion to cells, ingestion of particles by cells, and destruction of particles by lysosomal enzymes in phagolysosomes.

The percentage of monocytes increased in the 250 mg/L extract treatment, equivalent to 62.5 mg/L, 125 mg/L, and healthy fish (K). The DMRT test results showed that the infected sangkuriang catfish (0 mg/L) was significantly different from healthy fish (K) and the treatment with extracts (62.5, 125, and 250 mg/L). The results of this study indicated that the extract could increase the percentage of sangkuriang catfish monocytes. Maftuch (2007) states that in the inflammatory process, when tissue damage by microbial infection or antigen-antibody reactions occurs, it can increase monocyte production to two times, and monocytes mature into macrophages more quickly, so they can immediately go to the inflamed tissue.

Monocytes 9.4 -10.7 μ m in diameter, round or protruding, an eccentric nucleus exposed chromatin in the cytoplasm. Monocytes are highly basophilic and often have clear vacuoles in the cytoplasm. Monocytes function as precursors of macrophages and are active phagocytes. Lysosomal hydrolysis enzymes digest incoming material. Monocytes appear as irregular cells, have large nuclei and contain lots of chromatin. Monocytes migrate following the migration of granulocytes caused by inflammation (Hrubec *et al.*, 2000).



Monocytes function as macrophage precursors, monocytes–macrophages are active phagocytes. Incoming foreign material will be killed and digested by lysosomal hydrolysis enzymes. Monocytes–macrophages migrate following the migration of granulocytes stimulated by inflammation (Clauss *et al.*, 2008). The increase in the percentage of monocytes during the infection process in the fish's body is caused by monocytes pushing and destroying foreign objects that enter, such as bacteria. Monocytes are cells in the bloodstream and develop into macrophages. When monocytes are activated, macrophages have a more vital phagocytic capacity than neutrophils, even though the number of granulocytes is more significant (Irianto, 2005).

DMRT test results showed that the percentage of lymphocytes did not differ between all treatments. However, there was an increasing trend in fish treated with 250 mg/L. The average rate of lymphocytes that increased due to the administration of jeruju leaf extract indicated that the humoral immune response of sangkuriang catfish had recovered to a good condition, resulting in the process of resistance to bacterial infection with the formation of antibodies. Lymphocytes in the bloodstream migrate to the lymph or into the spleen. When they meet a foreign object, the lymphocytes will develop and undergo mitosis to become plasma cells that function as antibodies (Sadikin, 2002).

According to Hrubec *et al.* (2000), lymphocytes are essential cells in the immune response consisting of B cells and T cells. B cells function as cells that produce antibodies, while T cells function to control the immune response. The number of fish lymphocytes under stress conditions due to infection will decrease and trigger lymphopenia (Olga, 2009).

Lymphocytes are small cells with large nuclei and contain little cytoplasm—lymphocytes found in fish, namely small and large lymphocytes. The diameter of small lymphocytes ranges from 4.6 to 5.0 μm , dark purple; the cytoplasm of lymphocytes is dark blue and often consists of only a thin circle surrounding the nucleus. Large lymphocytes have a diameter of 5.7 to 6.4 μm , larger round nuclei, and a more exposed chromatin pattern than small lymphocytes. The cytoplasm of large lymphocytes is deeper and more basophilic than that of small lymphocytes. Small lymphocytes and large lymphocytes have a high N:C ratio. Lymphocytes generally have cytoplasmic pseudopods and azurophilic cytoplasmic granules (Hrubec *et al.*, 2000).

The results showed that the number of lymphocytes was lower in sangkuriang catfish infected with *A. hydrophila* bacteria. Bijanti (2005) explains that the decrease in lymphocyte cells is influenced by the presence of foreign antigens (such as bacteria), causes the immune system to be disturbed, and causes the number of lymphocytes to decrease. The decrease in lymphocytes is because lymphocytes are the body's first line of defense against infection (Alamanda *et al.*, 2007).

The basophils percentage decreased in the treatment of 62.5 mg/L equivalent to healthy fish, while in infected fish, there was an increase of basophils equivalent to the treatments of 125 mg/L and 250 mg/L. The results of the DMRT test showed that the treatment of healthy fish (K) and 62.5 mg/L of jeruju leaf extract was significantly different from the treatment with extracts (0, 125, and 250) mg/L. These results showed that jeruju leaf extract 62.5 mg/L reduced the number of basophils in infected fish and was equivalent to healthy fish.

Basophils are coarse basophilic granules in the cytoplasm with a round, cloudy nucleus. Basophil cells have a weak phagocytic ability and are often found in acute infectious conditions. Basophils are smaller in diameter than neutrophils, about 9-10 μm , making up 1% of the total white blood cells. Basophils have many cytoplasmic granules that cover the nucleus and contain heparin and histamine. In tissue, basophils become "mast cells." Basophils have immunoglobulin G (IgG) attachment sites, and their degranulation is associated with histamine release. Its function plays a role in allergic responses (Hoffbrand & Pettit, 1996).

The percentage of eosinophils showed an increase in fish given extract 125 mg/L, followed by 250 mg/L and healthy fish (K). The results of the DMRT test showed that the treatment of infected fish (0 mg/L) was significantly different from the treatment of fish with extracts (125 and 250) mg/L and healthy fish. Meanwhile, the extract treatment of 62.5 mg/L



was not significantly different. These results showed that jeruju extract 125 and 250 mg/L increased the number of fish eosinophils given the extracts and showed almost the same amount as healthy fish. According to Ramnik (2003), the estimated time for the formation of eosinophils is 2-6 days, and then the formed eosinophils are activated and can increase the fish's body defense. The lifespan of circulating eosinophils is 6-12 hours and will then survive in the tissue for two weeks. The results of clinical symptoms and leukocyte differential showed that *jeruju* leaves extract could cure sangkuriang catfish infected with *A. hydrophila*. *Jeruju* leaves extract can stimulate the performance of sangkuriang catfish leukocyte cells as an immune response to bacterial infection.

CONCLUSION

The findings concluded that there were differences in fish behavior and clinical symptoms in the speed of healing due to *A. hydrophila* infection from each treatment. The differential results of sangkuriang catfish leukocytes increased for the percentage of neutrophils and monocytes at 250 mg/L and eosinophils at 125 mg/L. Both of these concentrations were effective concentrations to stimulate differential leukocytes of sangkuriang catfish infected with *A. hydrophila*.

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