The effect of *Ocimum sanctum* L. crude extract on haematology of *Cyprinus carpio* infected by *Aeromonas hydrophila*

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Abstract. *Cyprinus carpio* L. is one of the most important freshwater fish and has been intensively cultivated. However, microbial infection become restricting component that can cause economic loss in carp production and the use of commercial antibiotics for therapy produces adverse side effects. This study was conducted to evaluate the potential of *Ocimum sanctum* L. crude extract on haematology of *Cyprinus carpio* against *A. hydrophila* infection. For this purpose, fish that have been infected were immersed in different doses of *Ocimum sanctum* L. crude extract (50, 150, 250, 350 ppm) and compared with the control group. After 1 week of immersion, total erythrocytes, total leukocytes, and deferential leukocytes (neutrophils, monocytes and lymphocytes) were recorded. The result showed that significant increase of total erythrocytes was obtained at the concentration of 350 ppm *Ocimum sanctum* L. crude extract. Furthermore, decreasing of the total leukocytes about 55.06×10³ to 25.77×10³, also decreasing deferential leukocytes (monocytes 40%, neutrophils 31% and lymphocytes 49% from the positive control). These results suggested that 350 ppm of *Ocimum sanctum* L. crude extract is beneficial to enhance the haematological status of common carp against *A. hydrophila*.

1. Introduction

Carp (*Cyprinus carpio*) is one type of freshwater fish that has important economic value [1]. Nowadays, to increase the production, carp are intensively cultivated [2,3]. However, in intensive cultivation with limited environmental conditions, high stocking density, overfeeding problem, as well as inappropriate water quality management can disturb environmental balance, so that fish become stressed and susceptible to disease [4,5].

One of the major bacterial pathogens is *Aeromonas hydrophila*. *Aeromonas hydrophila* is a ubiquitous gram-negative rod-shaped bacterium which is commonly isolated from fresh water ponds and which is a normal inhabitant of the gastrointestinal tract [6]. *A. hydrophila* is a causative agent of haemorrhagic septicemia on carp or known as “Motile Aeromonas Septicemia” which now become a serious problem in fish culture [7,8]. This disease often attacks freshwater fish and can cause outbreaks disease with a high mortality rate (80 – 100%) within 1 – 2 week [9,10].

Treatment of bacterial attacks is generally done by antibiotics. However, the use of antibiotics can cause side effects to both pathogen and the fish. Continuous use of antibiotics can cause pathogenic organisms become resistant, so the use of antibiotics becomes ineffective [11–13]. Whereas in fish it
can cause bioaccumulation, and cause carcinogenic effects (cancer-causing) if consumed by humans [14–16]. Therefore, several alternative strategies to antimicrobial usage have been proposed, including the use of medicinal plant as biological control agents [17,18].

*Ocimum sanctum* L. is an aromatic shrub in the basil family Lamiaceae that can be found easily in Indonesia. It contains many compounds, such as flavonoids, alkanoids, saponins, tannins, phenols, anthocynins, flavonoids, triterpenoids, tannins which are potential sources for therapeutic importance [19,20]. Methanolic crude extracts of *Ocimum sanctum* L. showed significant activity against *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Edwardsiella tarda* [21]. *Ocimum sanctum* also acts against *Klebsiella* spp, *Anthrobacter globiformis*, *Bacillus megaterium*, *Escherichia coli*, *Pseudomonas* spp. *Staphylococcus aureus*, *Staphylococcus albus* and *Vibrio* [22,23]. Leaves extract of *O. sanctum* affected both specific and non-specific immune responses and disease resistance against fungal and bacterial infection [24]. Many results proves that *Ocimum sanctum* L can act as excellent antimicrobial agent against many microbes. This study was conducted to evaluate the potential of *Ocimum sanctum* L. crude extract on haematology of *Cyprinus carpio* against *A. hydrophila* infection.

### 2. Materials and methods

#### 2.1 Fish

The common carp, *C. carpio* were obtained from Reproduction Laboratory, Faculty of Fisheries dan Marine Science, University of Brawijaya, Malang, Indonesia. *C. carpio* with 7-12 cm in length were used as many as 150 fish. Fish were kept under the same environmental conditions and placed in 150 L aquarium for a week as an acclimatization period to the laboratory condition and fed with a commercial diet.

#### 2.2 Plant extract

*Ocimum sanctum* L. were collected from Vegetable Market, Sengkaling, East Java, Indonesia. All parts of this plant were washed, dried at room temperature and ground. One kilogram powdered sample was macerated in 70% ethanol for 3 × 24 hours at room temperature. The resulting extract was concentrated over a rotary vacuum evaporator and then frieze-dried.

#### 2.3 Aeromonas hydrophila

*Aeromonas hydrophila* (AT118) was obtained from Control Fish Quarantine Center Quality and Safety of Fishery Products Class I Surabaya.

#### 2.4 Experimental design

After a weeks of acclimatization period, healthy *C. carpio* were infected by intraperitoneal injection (IP) with pathogenic *A. hydrophila* diluted in distilled water (10⁷ CFU) and were transferred into the same tanks. The fish were maintained for 1 week and the clinical symptoms after infected with *A. hydrophila* bacteria were observed.

After infection with *A. hydrophila*, blood samples of infected carp were taken and counted for total erythrocytes, total leukocytes and differential leukocytes. After that, the infected fish were then given an immersion treatment with different dose of *Ocimum sanctum* L. extract; of 50 ppm (A), 150 ppm (B), 250 ppm (C) and 350 ppm (D) in 15 L aquarium (10 fish/aquarium) for 24 hours. There were three replicates for each treatment arranged randomly. In this study, 2 comparison controls were used, negative control and positive control, negative control as sample treatment without bacterial infection and without giving basil crude extract, while the positive control as the sample treatment with infection *A. hydrophila* bacteria, the negative and positive controls for comparison only. During immersion, each aquarium were aerated to increase the dissolved oxygen content. After immersion for 24 hours, fish were transferred to another aquarium and maintained for 1 week. Then observed for total erythrocytes, total leukocytes and differential leukocytes in fish for 2 days once.
2.5 Haematology, biochemistry and serum collection

Blood was collected from the caudal vein using a 1 mL spuit disposable contains Na Såtrat 3.8% as anticoagulan. Two fish were randomly collected from one of the replicate aquarium for haematological assays: total erythrocytes, total leukocytes and differential leukocytes. Total erythrocytes were counted with a haemocytometer using Hayem’s RBC diluting fluid [25]. Total leukocytes were counted with a haemocytometer using Türk’s solution. Differential leukocytes were counted from one or more blood slides of each experimental group. The blood smear was fixed with methanol for 5 min by immersing the entire microscope slide with the blood smear into methanol. After that, the blood smear were removed from methanol and were allowed to air dry. Then the blood smear were colored by inserting them into the 10% Giemsa solution for 30 minutes, washed, then allowed to air dry. Blood smears that have been stained were observed and counted on the under a microscope with 1000x magnification until it reaches 100 leukocytes. Leukocytes were identified as lymphocytes (LYM), monocytes (MON), eosinophils (EOS), and neutrophils (NEU) or other leukocytes [26].

2.6 Statistical Analysis

Statistical analysis was performed by one way ANOVA. Analysis of variance (ANOVA) was used to determine the overall significance of differences among samples and a post hoc Tukey test for multiple comparisons between groups of samples assembled according to the significant factors and continued by orthogonal polynomial test. All the statistical analyses were carried out by using SPSS program version16. Values are expressed as mean standard deviation.

3. Results and discussion

3.1 Red Blood Cell Count

The RBC count was significantly increased in all the treatment groups compared with control group including post challenge period (Fig. 1). Based on the results of the study, the average of red blood cell count in the negative control K (-) was 218.4 x 10^5 cells/ml and the Positive control K (+) was 135.33x 10^5 cells/ml. While in treatment A (50 ppm) was 176.73 x 10^5 cells/ml, B (150 ppm) was 165.53 x 10^5 cells/ml, C (250 ppm) was 189.97 x 10^5 cells/ml and D (350 ppm) was 211.86 x 10^5 cells/ml. RBC count in treatment D (350 ppm) included in healthy or normal fish erythrocytes count, because it was still in the range of total erythrocytes count at negative control K(-). The increase in the number of erythrocytes in each treatment showed that the crude Ocimum sanctum extract given at different doses was able to treat fish infected with A. hydrophila bacteria. Similarly, [27] observed that adding Ocimum sanctum increasing the red blood cell of Labeo rohita and boosting the defence mechanisms and protection against Aeromonas hydrophila. [28] Amirkhani and Farid observed that Basil (Ocimum basilicum) supplementation diet containing ethanol extract can prevent infection Aeromonas hydrophila in common carp (Cyprinus carpio). Ocimum sanctum commonly known as holy basil or tulsi is considered as a sacred plant in hindu belief and known a queen of herbs. Crude extract of basil in the process of treating bacterial diseases causes death in A. hydrophila because of its chemical composition, it is suspected that the active substances that can act as antibacterial is ethanol and flavonoids.

3.2 White Blood Cell Count

Based on the results of the study, the average of white blood cell count (WBC) in common carp in the negative control K (-) was 8.60 x 10^3 cells/ml and the positive control K (+) was 15.02 x 10^3 cells/ml. While in treatment A (50 ppm) was 19.84 x 10^3 cells/ml, B (150 ppm) was 10.65x 10^3 cells/ml, C (250 ppm) was 11.44 x 10^3 cells/ml and D (350 ppm) was 8.83 x 10^3 cells/ml (Fig. 2). Based on the results of the average number of show that the average number of leukocytes of C. carpio in treatment D (350 ppm) is the lowest at 8.83 x 10^3 cells/ml, this value is in the normal range because it relates to the number of leukocytes in K (-) which is 8.60 x 10^3 cells/ml, but it can be seen in treatment C (250 ppm) the average of leukocyte increased, presumably because leukocytes fight foreign materials (Aeromonas hydrophila). Total leukocytes in treatment D was the lowest, presumably because the crude leaf extract of O. sanctum inhibit and kill pathogens because it is suspected that the active substance that acts as an...
The results in this study are supported by research conducted by [29]. The highest antibacterial effect of ethanolic *E. hirsutum* extract may be due to its high content flavonoids, tannins and steroids. In fact, this compound is known for its strong antimicrobial activity. It was indicated that the ethanolic extract of *E. hirsutum* inhibited growth of pathogens on agar plates as well. Effect of antibacterial activity was observed in *Ocimum sanctum*. The crude aqueous extract of leaf possesses some antibacterial and immunomodulatory. The ethanolic extracts from the leaves showed better activity against the β-lactamase producing methicillin-resistant *Staphylococcus aureus* and *Aeromonas hydrophila* strains [30].

3.3 Differential of Leukocyte

The percentage of neutrophils in treatment A (50 ppm) was 9.44% which showed the highest value compared to other doses. In those treatment, fish infected with *A. hydrophila* became stress and got the loger inflammation. While, the lowest percentage of neutrophils was in treatment D (350 ppm) which was 4.00% (Fig. 3). This value is to be in the normal range because it corresponds to the percentage of neutrophils at K (-) which is 4.67%. The number of neutrophils will increase in the event of infection or attack by bacteria, because neutrophils move by diapedesis through capillary pores and by chemotaxis to damaged tissue areas. In the fish, the oxygen radicals are at the destruction of bacterial invaders. Studies on neutrophil activity clearly showed the enhancing effect of *O. sanctum* leaf extract on neutrophil respiratory burst (RB) activity [31].

Fish given treatment A (50 ppm) showed the percentage of monocytes and lymphocytes that was close to the results of the positive control K (+) (Fig. 4 and 5). However, when compared to other treatments, treatment D (350 ppm) showed the lowest monocyte percentage, which was 9.50%, when compared to the negative control K (-) of 10.00%. While, the percentage of lymphocyte in treatment D (350 ppm) was 14% compared to the negative control K (-) 14.3%. In the event of infection by a foreign materials, monocytes will move quickly from the blood to the infected area to carry out phagocytosis. The ability of monocytes to penetrate the walls of capillary blood vessels, enter the tissue and differentiate into macrophage cells. Similarly, the explanation by [32], in whole blood culture, lymphocytes, thrombocytes and erythrocytes did not show phagocytosis but neutrophil and monocyte like cells showed active phagocytosis.

The increment doses of *O. sanctum* leaf extract showed that the percentage of phagocytic activity in fish infected with *A. hydrophila* was getting lower. This is presumably because the immune system in *C. carpio* more active to kill *A. hydrophila* bacteria with the addition of basil leaf extract to the highest dose (350 ppm). The lymphocyte and monocyte plays an important role in primary defense mechanism because of their engulfing nature against foreign particles. Monocytes kill bacteria by producing reactive nitrogen intermediates (RNIs) and reactive oxygen intermediates (ROIs) and through the action of phagolysosomal enzymes [33].

![Figure 1](image-url)  
**Figure 1.** Effect of *O. sanctum* leaf extract on red blood cell count (RBC) during pre and post challenge. Indicates significant difference (P < 0.05).
Figure 2. Effect of *O. sanctum* leaf extract on white blood cell count (WBC) during pre and post challenge. Indicates significant difference (P < 0.05).

Figure 3. Effect of *O. sanctum* leaf extract on percentage of neutrophil during pre and post challenge. Indicates significant difference (P < 0.05).
Figure 4. Effect of *O. sanctum* leaf extract on percentage of Monocyte during pre and post challenge. Indicates significant difference (P < 0.05).

Figure 5. Effect of *O. sanctum* leaf extract on percentage of lymphocyte during pre and post challenge. Indicates significant difference (P < 0.05).

4. Conclusion
Administration of crude extract of basil leaves (*O. sanctum*) with different doses gave a significant effect on the hematological response of *C. carpio* and gave an effect on total erythrocyte, total leukocytes and leukocyte differential after infected with *A. hydrophila*. The best dose of extract to increase immunity in *C. carpio* against bacterial infection *A. hydrophila* is 350 ppm.
5. References

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