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Cyprinus carpio hematological changes after dietary *Coffea canephora* leaf extract for *Aeromonas hydrophila* infection

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ABSTRACT

Coffea canephora or robusta coffee was known as a medicinal plant that admitted various biological properties. However, its application as a medicinal plant in fish disease treatment has not been reported. This study aimed to investigate the effect of dietary robusta coffee leaf (RCL) extract on the survival rate and hematology of *Cyprinus carpio* infected by *Aeromonas hydrophila*. Two hundred fish were randomly divided into five treatment groups consisting of uninfected fish (UF), infected fish (IF), and a diet supplemented with different concentrations of RCL extract (30%, 40%, and 50%). The negative control group was injected by NaCl 0.9% and other treatment groups were injected by *A. hydrophila*. Feeding treatment was carried out for 7 days after clinical symptoms appeared. The results showed that RCL extract increases the survival rate and hematological parameters (number of erythrocytes, hemoglobin, and lymphocytes) significantly ($P<0.05$). The highest survival rate (100%) was recorded in groups that accepted 50% of RCL extract. This current study indicates that RCL extract could be considered a potent medicinal plant for treating fish disease caused by *A. hydrophila* at the optimum dose of 50%.

Introduction

Motile Aeromonas Septicemia (MAS) is a fish disease caused by motile members of the genus *Aeromonas*. The three most common species of Aeromonas are known to be responsible for fish infection with hemorrhagic septicemia syndrome were *A. hydrophila*, *A. sobria*, and *A. caviae* (Hu *et al.*, 2012). Meanwhile, *A. hydrophila* is recognized as the most deadly species among motile Aeromonas species (Kozińska and Pkala, 2012). It can infect almost all of the freshwater fish including carp and cause mass mortality in a short period of infection (Esteve *et al.*, 1995; Sarkar and Rashid, 2012). The infection of *A. hydrophila* generates various clinical symptoms including dermal ulceration, hemorrhage, inflammation, tail or fin rot, exophthalmia and abdominal distension (Cipriano *et al.*, 1984). In preventing the mass mortality caused by *A. hydrophila* infection, antibiotics were used as the agent of fish treatment in aquaculture. However, its excessive and long-term used induced some side effects such as increasing resistance to the bacterial pathogen and the presence of residual antibiotics in the fish product (Rasul and Majumdar, 2017). The adverse impact of antibiotic application in fish treatment has been induced an exploration the alternative drugs which are safer and eco-friendly (Awad and Awaad, 2017).

Phytotherapy is an ancient mankind healthcare method that has been used for long time before the chemical drugs were known. This therapy utilized many parts of the herbal or medicinal plants for disease treatment. In recent years, phytotherapy has become an interesting topic in aquaculture as an alternative drug for shifting the chemical drug such as an antibiotic (Raman, 2017). Phytotherapy becomes an alternative treatment method because it

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is safe, eco-friendly, easily obtained, and well applied in fish diseases.

Robusta coffee (*Coffea canephora*) is a species of coffee plant that is mostly cultivated in Indonesia (Rosiana *et al.*, 2018). Besides the coffee bean, the coffee plantation also produced coffee leaves from the pruning process. Pruning is regularly needed in coffee plantation management to optimize the production of coffee fruit and maintain the coffee plant. However, this process generated a large amount of coffee leaf waste which can be an environmental pollution source if it does not properly process (Martinez *et al.*, 2019). Some previous studies revealed that coffee leaves contain various phytochemical contents, such as alkaloids (caffeine, trigonelline, and theobromine), flavonoids (catechins, quercetin, and kaempferol), terpenes (kahweol, cafestol, and ursolic acid), xanthonoids (mangiferin and isomangiferin), tannins, phenolic acids (caffeic acid, chlorogenic acids, and p-coumaric acid), and carotenoids, which are biologically active as anti-bacterial, anti-oxidant, and anti-inflammatory (Chen, 2018; Chen *et al.*, 2019; Kenconojati *et al.*, 2019). Due to this fact, robusta coffee leaves are considered to become a promising natural product which can be used for replacing chemical drugs, especially antibiotics. To our knowledge, the application of robusta coffee leaf waste as phytotherapy agent for fish disease treatment has not been studied yet. Hence, the present study was carried out to examine the effect of dietary robusta coffee leaf (RCL) extract on the survival rate and hematology of common carp (*Cyprinus carpio*) infected by *A. hydrophila*.

Materials and Methods

Location and time of research

This research was conducted at Teaching Farm and Instrument Laboratory of Campus Banyuwangi Universitas Airlangga from January to April 2019.

Fish collection and maintenance

A total of two hundred common carp were purchased from the fish farm at Genteng, Banyuwangi, Indonesia. The fish were divided into 20 glass tanks with 70 L capacity. All fish were acclimatized for one week before the treatment. The fish were fed with commercial feed (FF-999, PT. Central Proteina Prima, Indonesia) three times daily as much as 4% of body weight. Water quality maintenance was carried out by the removal of residual feed and fecal matter once a day. There were no clinical signs that found during the acclimatization period.

Preparation of experimental diets

The experimental diets were arranged the using spraying method as reported by Dotta *et al.* (2018) with some modifications. The commercial diet (FF-999, PT. Central Proteina Prima, Indonesia) contained 40% crude protein as the basal diet was used in this study. RCL extract was obtained by maceration as described by Kenconojati *et al.* (2019). The concentration of RCL extract used is as follows 30%, 40%, and 50%. The extract was diluted in 10 mL of ethanol. The mixture was homogenized using a vortex and sprayed on feed pellets. Then, it dried using oven at 60ºC overnight. No extract was added to commercial diet for uninfected (UF) and infected fish (IF) groups.

Experimental design

This study was conducted in a completely randomized design (CRD). All fish were divided into five groups, consisting of uninfected fish (UF), infected fish (IF), and feeding treatment with RCL extract in various concentrations (30%, 40%, and 50%). Fish in UF groups were injected with NaCl 0.9% solution and other treatment groups were injected with *A. hydrophila* suspension (0.1 mL) at a concentration of 10^6 CFU/mL intramuscularly. Isolate *A.hydrophila* was obtained from Balai Perikanan Budidaya Air Payau Situbondo. Feeding treatment was performed 24 hours after injection for 7 days. UF and IF groups were fed a commercial diet without RCL extract addition. Fish were fed as much as 4% of total body weight three times daily. Water quality parameters including temperature, pH, dissolved oxygen, and ammonia were measured during the maintenance. Siphon was carried out once a day for removing residual feed and fecal matter. During the maintenance, the water quality was in the range of 24-28°C, pH 7-8, dissolved oxygen 5 mg/L, and ammonia 0-0.5 mg/L.

Hematological analysis

Blood samples were taken two times, before and 7 days after feeding treatment. Blood samples were collected from the caudal vein using a syringe containing a drop of 1% EDTA solution. The collected blood was divided into four different analyzes: total erythrocytes and total leucocytes were counted manually using a Neubauer hemocytometer as described in Harikrishnan *et al.* (2003), hemoglobin concentration was measured with the Sahli method (van Lerberghe *et al.*, 1983), the number of the differential leucocytes were classified and calculated using May-Grunewald-Giemsa techniques which thin blood films were air dried, fixed in methanol and stained by Romanowsky stain (Blaxhall and Daisley, 1973).

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Disease resistance

Fish mortality was recorded during the treatment and survival was calculated at the end of the experiment according to the formula (Dotta *et al.*, 2018):

$$\% \, Survival = \frac{Number \; of \; surviving \; fish}{Number \; of \; infected \; fish} \times 100$$

Data analysis

Data analysis was performed using SPSS software (SPSS version 20 for Windows). Results are presented as mean and standard error. The difference between the control and treatments were analyzed using a one-way analysis of variance (ANOVA) followed by the Duncan multiple range test. The significant differences were considered when P<0.05.

Results

Hematological parameters

Total erythrocytes of fish in 1st-day post-challenge by *A. hydrophila* (before feeding treatment) significantly decreased (p<0.05) compared to uninfected fish groups (Figure 1). After 7 days of feeding treatment, the number of erythrocytes increased in almost all the treatment except in infected fish groups. Dietary of 50% RCL extract showed the greatest number of erythrocytes which is similar to the uninfected fish group. This result was significantly different (p<0.05) among the treatment.

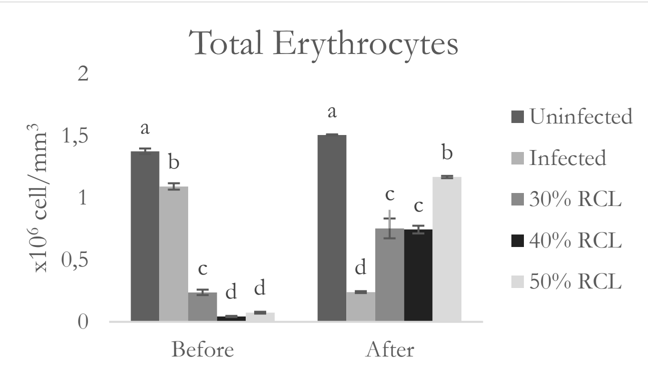

Figure 1. Total erythrocytes (x10^6 cell/mm^3) of *C. carpio* infected with *A. hydrophila* before feeding and 7 days after feeding with different levels of RCL extract. The different superscript indicates a significant difference among the treatment (P<0.05)

Similar to the number of erythrocytes, *A. hydrophila* considerably decreased the concentration of hemoglobin in the 1st-day post-challenge significantly reduced (p<0.05). After 7 days of feeding treatment, the level of hemoglobin changed. Hemoglobin levels decreased in the infected fish group, 30%, and 40% of RCL extract supplemented diets feeding groups. However, feeding with 50% of RCL extract on diets could enhance the hemoglobin levels (Figure 2).

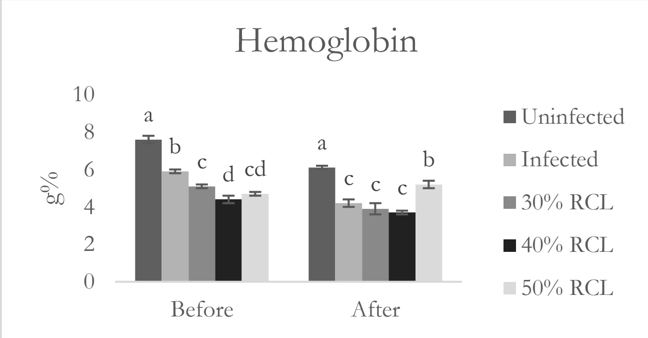

Figure 2. Hemoglobin of *C. carpio* infected with *A. hydrophila* before feeding and 7 days after feeding with different levels of RCL extract. The different superscript indicates a significant difference among the treatment (P<0.05)

Infection of *A. hydrophila* also influenced the number of leucocytes in fish. Total leucocytes of infected fish enhanced one day after the challenge (p<0.05). At the end of feeding therapy, the best leucocyte improvement happened in the diet of 50% RCL extract (Figure 3).

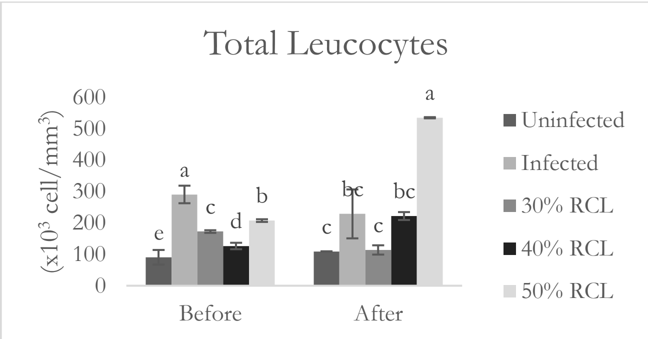

Figure 3. Total leucocytes (x10^3 cell/mm^3)of *C. carpio* infected with *A. hydrophila* before feeding and 7 days after feeding with different levels of RCL extract. The different superscript indicates a significant difference among the treatment (P<0.05)

The number of differential leucocyte parameters is presented in the Table 1. The number of monocytes, lymphocytes, and neutrophils showed significant differences when fish were treated with an RCL extract diet after 7 days of feeding treatment.

Table 1. Differential leucocytes parameters counted in *C. carpio* infected with *A. hydrophila* and fed with different level of RCL extract.

Treatment	Monocytes (%)	Lymphocytes (%)	Neutrophils (%)
Uninfected	16.50±0.5[c]	59.50±0.5[a]	24.00±1.0[c]
Infected	40.00±1.0[a]	22.50±1.5[d]	37.50±0.5[a]
30% RCL	25.50±1.5[b]	54.00±1.0[b]	20.50±0.5[d]
40% RCL	16.00±0[c]	53.50±0.5[b]	30.50±0.5[b]
50% RCL	26.50±0.5[b]	46.00±1.0[c]	27.50±1.5[b]

Letters in the column indicate a significant difference among treatments (P<0.05)

Disease resistance

The survival percentage of common carp during the treatment is shown in Table 2. The result presented that no fish died in the uninfected fish group, but the highest mortality occurred in the infected fish group (55±18.01%). Infected fish treated with 50% of RCL extract showed higher longevity compared to the other treatment significantly (p<0.05).

Table 2. The survival rate of *C. carpio* at the end of the feeding treatment.

Treatment	Survival Rate (%)
Uninfected	100±0[a]
Infected	55±20.82[b]
30% RCL	60±16.33[b]
40% RCL	70±21.60[b]
50% RCL	100±0[a]

Different letters indicate a significant difference among the treatments (P<0.05)

Discussion

Hematological parameters changes can be used as the indicators of fish health status. Changing the blood level and damaging fish tissue usually happen during periods of the contaminant exposure, for example bacterial infection (Zorriehzahra *et al.*, 2010). In this study, the infection of *A. hydrophila* in common carp fish influenced the fish hematological profile such as erythrocytes, hemoglobin, leucocytes, and differential leucocytes. One day after infection, the number of erythrocytes and hemoglobin significantly decreased compared to the uninfected fish (P<0.05). This result can happen because *A. hydrophila* produced hemolysin and aerolysin during the infection (Rasmussen-Ivey *et al.*, 2016). These toxins known to have hemolytic activity caused damaging erythrocytes and exudation of the hemoglobin from capillaries (Kanai and Takagi, 1986). Different reactions happened with the total leucocytes of infected fish. Leucocytes are known as the white blood cells that have an important role in protecting the body against infectious agents. In our study, the leucocytes level of infected fish increased approximately 2 to 3 times higher compared to the uninfected fish. This case reflected the improvement of leucocytes production for enhancing the fish defense mechanisms against *A. hydrophila* infection. The similar case has been reported by Martins *et al.* (2008) and Harikrishnan *et al.* (2010).

The further study observed the percentage of lymphocytes, monocytes, and neutrophils which were also affected by *A. hydrophila* infection. In our result, the number of lymphocytes was reduced whereas the monocytes and neutrophil levels increased. These three components of leucocytes play important role in achieving protection against bacterial infection through a different mechanism. Their concentration also influenced each other. Neutrophils and monocytes were a type of leucocytes that are involved in antibacterial defense through the phagocytosis mechanism (Kumar *et al.*, 2018). Ellis (1999) explained that neutrophils will phagocytose pathogenic bacteria cell by producing reactive oxygen species (ROS), myeloperoxidase (MPO), lysozyme, and hydrolytic enzyme which help to lyse and digest the cell. Whilst, monocytes will become macrophages which are more powerful than neutrophils in phagocytosing particles. Lymphocytes have been reputed as immunocompetent cells that produced antibodies to attack the pathogen (Dangeubun and Metungun, 2017). The decreasing level of lymphocytes in blood circulation after infection can be suspected due to the re-dispensation of lymphocytes to lymphoid organs, high levels of cortisol in blood circulation, and cell eradication (Azimzadeh, 2016).

Robusta coffee (*C. canephora*) is well-known as a medicinal plant because of its various pharmacological activity. Many studies reported that all part of the coffee plant such as the seed, leaf, flower, and root can be used as a drug disease in human and veterinary medicine (Patay *et al.*, 2016). In our study, RCL extract was used as phytotherapy against *A. hydrophila* infection in common carp which applicate by supplemented on diet. Our result revealed the alteration of blood level parameters occurred after 7 days of feeding treatment with RCL extract. The number of erythrocytes and hemoglobin enhanced along with the concentration of RCL extract given. At the administration of 50% of RCL extract, the number of erythrocytes and hemoglobin was increased close to the total erythrocytes and hemoglobin level of uninfected fish group. The improvement of total leucocytes occurred after 7 days of feeding treatment. However, dietary of RCL extract in a concentration of more than 30% generated the production of leucocytes higher than the uninfected fish group ($109.01x10^3$ cell/mm^3). This result can be expected due to the side effect of the administration of RCL extract on diet. According to Kenconojati *et al.* (2019), RCL extracts are recognized as containing phytochemical compounds, for instance flavonoids, steroids, terpenoids, polyphenols, and saponin. The presence of these compounds is known to have an antibacterial activity that can be used as phytotherapy. Nevertheless, some of them, such as saponin, also have an anti-nutritional effect that is related to the fish's ability to nutrient absorption (Kumar *et al.*, 2012). Besides the bacterial infection and stress, nutritional deficiencies in fish could affect the fish hematological profile (Clauss *et al.*, 2008).

Conclusion

This study proved that the fish mortality caused by *A. hydrophila* can be pressed using RCL extract on a fish diet. The fish survival reaches 100% at the administration of RCL extract as much as 50% on diet. Meanwhile, using the RCL extract in a concentration of less than 50% did not cause alterations in fish longevity compared to the treatment without RCL extract. This result revealed that RCL extract performed the optimum bactericidal activity at 50% towards *A. hydrophila*. It could be mentioned that RCL extract could help in recovery from aeromoniasis disease.

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