International Journal of Engineering & Technology, 8 (1.9) (2019) 287-292

International Journal of Engineering & Technology

Website: www.sciencepubco.com/index.php/IJET

Research paper

The Effects of Ketapang (*Terminalia catappa*) Bark Crude Extract on Inhibition of *Aeromonas hydrophila* Growth and Blood Cells of the Infected Carp (*Cyprinus carpio*)

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Abstract

Research aimed to determine the effect of ketapang (Terminalia catappa) bark crude extract (KBCE) on inhibition of Aeromonas hydrophila growth and blood cells of the infected carp (Cyprinus carpio). Research was conducted by completely randomized design for both in vitro and in vivo. There were four (750, 850, 950, and 1,050 ppm) and three treatments (730, 750, and 770 ppm) were used for in vitro and in vivo experiments respectively. Both experiments were replicated three times. Diameter of inhibitory zone on A. hydrophila growth was main variable in experiments. Data were analyzed by analysis of variation (and LSD. based on in vitro, 750, 850, 950 and 1,050 ppm KBCE produced the average inhibitory zone diameter of 8.97, 10.21, 11.69 and 13.89 mm respectively. On in vivo, erythrocytes were 224.67x10, 332.67x10, and 417.00x10 cells/mm for 730, 750 and 770 ppm KBCE respectively. In case of leukocytes were 735.89x10, 684.78x10, and 652.67x10 cell/mm for 730, 750 and 770 ppm KBCE respectively. In addition, based on measurement of water quality, water had 24.0 – 25.9□C of temperature, 7.0 – 7.7 of pH, and 3.9 – 4.8 mg/L of DO.

Keywords: *Ketapang bark; Aeromonas hydrophila; carp; erythrocytes; leukocytes*

1. Introduction

Indonesia is a country which has vast potential of fisheries. It has been proven by the fisheries commodity exported to USA market which increased up to 25.19% during 2014-2015 (MMAF, 2015). The fisheries potential in Indonesia is obtained from wild fisheries and fish farming. However, the result of wild fisheries is getting lower. Thus, the products of fish farming are expected to support the fisheries export in the future. The types of fish farming in Indonesia can be divided into mariculture, pond culture, freshwater pond culture, and rice-fish field culture (Gusrina, 2008). A strategy is needed to develop sustainable fish farming.

The main problem faced by the fish farmer is fish diseases. Fish disease in pond is the result of mismatch interaction between fish, environmental condition and disease organisms (Snieszko 1973). This mismatch interaction can cause the fish becomes stressful. That condition reduces the fish defense mechanism which makes it easy to be infected with disease (Meyer, 1991). The infection of certain disease caused by bacteria is the main problem in fish farming process (Bondad et al., 2005).

A. hydrophila is a dangerous kind of bacterium for fish, particularly for unscaled fish (Turutoglu et. al., 2005). This pathogen can attack the fish under these following conditions; imbalanced nutrition contained in the fish fodder, high level of organic contained in the water environment, high level of water quality parameter fluctuation, secondary infection caused by parasite and genetic factor (low immunity of fish can be infected by bacteria) (Cipriano, 2001).

An attempt that has been done to cure the fish disease is the use of antibiotics which later on causing pathogenic bacteria resistance and antibiotic residues (Shak et al., 2011). Therefore, there is a need for alternative development of antibacterial substance made from medicinal plants, as known as phytochemical. One of the alternatives is using ketapang (Terminalia catappa) bark. This plant contains active compound known as flavonoid, it can kill or inhibit the growth of bacteria (P. Neelavathi et al., 2013). There were two main objectives in this research i.e. 1) to determine the effect and the minimum suitable dose of ketapang bark crude extracts to inhibit the growth of A. hydrophila, and 2) to determine the effects of ketapang bark crude extract on blood cells of the infected carp.

2. Materials and Methods

Research was conducted by completely randomized design. There were two experiments, such as in vitro and in vivo. Four treatments namely 750, 850, 950, and 1050 ppm were used for in vitro experiment. In case of in vivo, there were three treatments i.e.: 730, 750, and 770 ppm. Both in vitro and in vivo experiments were replicated three times. Inhibition of bacterial growth, A. hydrophila and changes of blood cells were investigated in this experiments. In addition, water quality of research media was also recorded to support the main objectives.

Petri dishes, micropipettes, blue tips, test tubes, Erlenmeyer flasks, inoculum needles, refrigerator, autoclave, incubator, laminar air flow, spectrophotometer, rotary vacuum evaporator, section set, digital scale, microscope, digital camera, beaker glasses, measuring cylinder, aquarium, aerator, thermometer, pH meter, DO meter, aquadest bottles, and funnel were used in experiments. There were some materials in the experiments such as: Carp (C. carpio) from Punten Batu East Java, fish with the size of 7-11 cm, A. hydrophila, ketapang (T. catappa) bark crude extract (KBCE), filter paper, ethanol 90%, Xylol, Formaldehyde 10%, label, alcohol 70%, tissue, Aquadest, NB (Nutrient Both), NaCl, paper discs, aluminum foil, gloves, mask, cotton, yarn, acetone, NA (Nutrien Agar), fish fodder (pellet), and fresh water.

2.1. Research preparation.

Equipment's, materials, room and related things were sterilized completely. The equipment and animal acclimatization were conducted for 7 days and A. hydrophila was obtained from Fish Quarantine and Inspection Agency Class I, Surabaya, East Java.

2.2. Preparation of Ketapang Bark Crude Extract (KBCE).

Ketapang bark crude extract used the modification method from Sumino et al. (2013), it was done by adding 96% ethanol solvent 96% with the proportion 1:5. Two and a half kilograms of the bark of ketapang tree were cleaned and dried by conventional aerating, then it was mashed up into powder. Two and a half kilograms of wet materials could produce one kg dry powder. Then, 500 g of ketapang bark powder was put into a jar and it was added with 2,500 ml of ethanol solvent 96% before homogenized. The jar was covered with aluminum foil to prevent ethanol evaporation. This maceration process was completed for three days which took a place in dark area. After three days, the maceration result was strained by using filter paper to separate the solution and its sediment. Then, the filtered substance was evaporated in order to obtain the pure extract from ketapang bark. This evaporation process was performed by using rotary evaporator under the temperature of 45° C and the speed of 80 rpm. After an hour of evaporation, there would be obtained thick pure extract.

2.3. Preparation of Media.

Nutrien Agar (NA) used in this research was 28g/l of OXOID. Then 2.8 g of NA was weighed and placed in Erlenmeyer containing 100 ml of aquadest. It was stirred at warm the temperature condition and placed on the hotplate until it was well mixed. Then, Erlenmeyer was covered with cotton and aluminum foil before NA was sterilized inside autoclave at 121oC for 15 minutes. The used media were cooled at ca. 30oC. Finally, it was poured into petri dish.

Nutrien Broth (NB). The dose of the NB was 8 gram/l. Then, 0.8 g NB was dissolved in Erlenmeyer containing 100 ml of aquadest. It was stirred until perfectly dissolved and turned into yellow. Erlenmeyer was covered tightly with cotton and aluminum foil. Then it was sterilized inside autoclave at 121oC for 15 minutes.

A. hydrophila bacterial culture. Inoculum needle was heated on Bunsen burner until burnt. When it turned cool, the inoculum needle was touched into the natural culture of A. hydrophila and then dipped into the NB solution that has been prepared. The NB solution was kept for 24 hours into an incubator at 37 oC. Next, Petri disc which contained the NA medium was prepared. After the solution became cloudy, inoculum needle was dipped into NB and scratched on the NA surface of Petri disc. Then it was dragged across the surface in zig-zag motion by using continuous streaking method, T or quadrant streaking method. The media of NA were incubated in the incubator at 30oC for 24 hours. The bacteria stock was sought to have 109 of density in the NB medium for next treatment.

Minimum Inhibitory Concentration (MIC) Test. The following explanations were the procedure of conducting MIC Test. First, nine test tubes containing 9 ml of NB sterile media were prepared. Then the crude extract of ketapang (Terminalia catappa) bark was putted into seven test tubes with different dose for each tube. The doses were 1000, 100, 10, 1, 0.1, 0.01 and 0 ppm. Each chemical tube was given one oose of bacterial isolates. Then, the media was incubated in the incubator at 32oC for 24 hours. Finally, the density of the media was checked and the absorbance value was measured by using spectrophotometer at 512nm wavelength.

Disc Test. The following explanations were the procedure of conducting Disc Test. Firstly, concentrate of KBCE was prepared to be used for disc test. Bacteria cultivation on NA media were done by taking bacterial culture from NB media with 109 density. Then, cotton swap was dipped and scratched completely on the whole media's surface. Six millimeters of sterile paper disc were soaked into KBCE for 15 minutes based on defined concentrate. Next, it was drained and placed on the agar's surface. The result could be read after incubation process at 300C for 24 hours and by using caliper to measure the diameter of inhibition zone which was formed around the paper disc. The space between paper disc and the edge of Petri disc could not be less than 15 mm. If there were more than one paper disc, the spaces between paper disc isn`t less than 24 mm.

Infecting Bacteria. Each aquarium was filled with 10 fish. Infecting A. hydrophila bacteria to the carp (C. carpio) was done by soaking the fish in the 30x30x30 cm3 aquarium equipped with aeration and 20 l water. Thus, the used dilution formula was described on equation 1. Infection process was performed for 24 hours. Then, the fish was removed to the fresh water and cured with KBCE for six minutes soaking. Each dose was 730, 750, and 770 ppm.

$$V1 \times N1 = V2 \times N2 \quad (1)$$

Red Blood Cells Measurement. The blood sample from carp was performed by using one cc disposable spuit which has been made wet with 3.8% Natrium citrate. According to Mones (2008), red blood cells measurement is conducted by using haemocytometer. The formula was used to measure red blood cells (on equation 2). There were some steps in measuring the amount of red blood cells. First, blood sample which has been mixed with anticoagulant was sucked by using RBC pipette up to the scale of 0.5. Then, Hayem solution was also sucked up until it showed 101 (dilution 1:200). Next, the pipette was shaken to completely mix the blood and Hayem solution. The first four drops were discarded and the fifth drop was dropped to hemocytometer. It was done based on the prediction that the fifth contained perfect mixture of blood and Hayem's solution. Then, hemocytometer was covered by cover glass and observed under the microscope by lowering down the condenser or diagram slowly. The red blood cells were observed by using 400x magnification and measured on the five squares in the chamber.

$$RBC = (A/N) \times (1/V) \times Fd \quad (2)$$

Notes: Red blood cell (RBC); Amount of red blood cells (A); Number of hemocytometer square observed (N); Volume of hemocytometer (V) and Dilution factor (Fd).

White Blood Cells Measurement. According to Mones (2008), white blood cells measurement was performed by using hemocytometer and counted by specific formula (on equation 3). There were some steps in measuring the amount of white blood cells. First, blood sample which has been mixed with anticoagulant was sucked by using WBC pipette up to the scale of 0.5. Then, Turk solution was also sucked up to 11 (dilution 1:20). Next, the pipette was shaken to completely mix the blood and Turk solution. The first four drops were discarded and the fifth drop was dropped to hemocytometer. It was done based on the prediction that the fifth contained perfect mixture of blood and Turk solution, so it would be easier to measure with microscope. Then, hemocytometers was covered by cover glass and observed under the microscope by lowering down the condenser or diaphragm slowly. The white blood cells were observed by using 400x magnification and measured on the 4 squares in the chamber.

$$WBC = (A/N) \times (1/V) \times Fp \quad (3)$$

Notes: White blood cell (WBC); Amount of white blood cell (A); Number of hemocytometer square observed (N); Volume of hemocytometer (V) and Dilution factor (Fd).
Data were analyzed by Analysis of variance (ANOVA). If there were significances between each treatment, Post hoc of Least Significance Different (LSD) is used to see differences for each treatment.

3. Result and Discussion

3.1. Minimum Inhibitory Concentration (MIC) Test.

Minimum Inhibitory Concentration (MIC) test was performed by using several doses of ketapang bark crude extract (KBCE). It was aimed to know the lowest dose of KBCE to inhibit the growth of A. hydrophila. Result showed that there was various absorbance values between each dose after being observed by using spectrophotometer at 512 nm wavelength. The results were described in the Table 1.

Table 1: Absorbance values of each dose based on MIC Test by Spectrophotometer

No.	Doses	Absorbance	Remarks
1	750 ppm	0.160	clear
2	800 ppm	0.530	clear
3	850 ppm	0.198	clear
4	900 ppm	0.095	clear
5	950 ppm	0.200	clear
6	1000 ppm	0.595	clear
7	1050 ppm	0.123	clear
8	Control (-)	0.358	Not clear
9	Control (+)	0.151	clear

Notes: A. hydrophila as Control (–); A. hydrophila was grown on the medium of 100% ketapang bark crude extract (KBCE) as Control (+)
Based on the spectrophotometer result in tube 1 with the dose of 750 ppm, the absorbance value was 0.160. That value was close to value of the positive control (0.151). The result of MIC test was observed by looking at the clear color change for the first time which showed that the positive control was closed to 750 ppm. It indicated that the dose of 750 ppm could inhibit the growth of A. hydrophila.

3.2. Antibacterial Potency of Ketapang Bark Extract Using Disc Test.

Based on the observation, inhibition potency test of A. hydrophila by using ketapang bark crude extract (KBCE), the clear zone could be seen in the Figure 1.

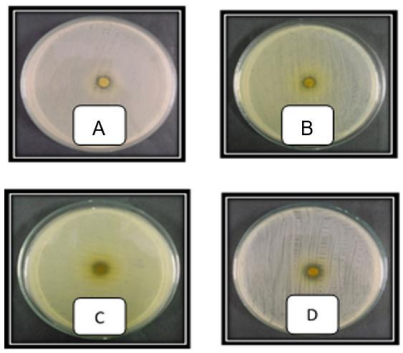

Fig. 1: Inhibition of Ketapang Bark Crude Extract on A. hydrophila based on Disc Test, A, B, C and D were treatment doses of KBCE 750, 850, 950, and 1,050 ppm respectively.

The clear zone as shown in Figure 1 indicated that there was no bacteria growth around the disc which has been soaked by using different doses. The clear zone diameter in each petri dish was effected by the amount of dose for each treatment. The highest dose produced the biggest clear zone. It was supported by a statement from Riskitavani and Kristanti (2013), ketapang is known to have medicinal compound such as flavonoid, tannin, triterpenoid/steroid, resin and saponin which categorized as antibacterial compound. In accordance to the statement from Harborne (1998) and Thompson et al., (2006), based on phytochemical test, bark positively contained phenolic, tannin, flavonoid, alkaloid, terpene and steroid.
Based on the observation, the average diameter of the smallest inhibition zone in KBCE at 750 ppm was 8.97 mm, meanwhile the average diameter of the biggest inhibition zone was at 1,050 ppm for 13.89 mm. Analysis of variance (ANOVA) test showed significant difference between treatments ($p<0.01$).

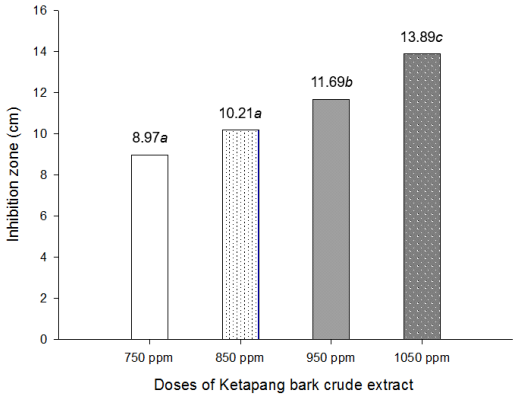

Fig 2: The Relation between doses of Ketapang bark crude extract and the diameter of inhibition zone of A. hydrophila. Data are represented as mean (n = 12). Different letters indicate significant differences (P < 0.01) among four treatments determined by one-way ANOVA and continued by LSD 5%.

Based on Figure 2, it could be seen that the use of ketapang bark crude extract (KBCE) could inhibit the growth of A. hydrophila; the average diameter of inhibition zone ranged between 8.97 and 13.89 mm which indicated that KBCE had strong antibacterial activity. Related to Ismaini (2007), he classifies the inhibition zone of antibacterial activity into low activity (< 5 mm), medium activity (5 – 10 mm), strong activity (11 – 20 mm), and very strong activity (> 20 mm), KBCE can be classified as medium (750 ppm) and strong activities (850, 950 and 1050 ppm).

Strong inhibition potency of KBCE on the growth of A. hydrophila caused by ketapang bark (T. catappa) contained active materials such as saponins, triterpenes, tannins, alkaloids, iridoid glycosides, and flavonoids. The roles of those chemical compounds were antibacterial (saponin), hemostatic and astringency (tannin), analgesic (alkaloids), anti-inflammatory (iridoid glycosides compound), and antioxidants and anti-inflammatory (flavonoids) (Masuda et al., 1999).

Phenol compound and its derivatives (flavonoids) were some of the antibacterial that worked by interfering the function of the cytoplasmic membrane. At low concentrations, it could damage the cytoplasmic membrane which later on could cause the leak of important metabolites that inactivated bacterial enzyme system. Meanwhile, at high concentrations it could damage the cytoplasmic membrane and precipitated the cell protein (Volk and Wheeler, 1984).

Low inhibitory zone indicated that there has been dose of materials resistance towards A. hydrophilia. Therefore, that dose has not been able to damage or kill the cells of the bacteria. According to Pelczar and Chan (1986), the resistance that occurs in an organism towards the concentration of a substance is a natural mechanism to defend a life. Based on the length of inhibition time which was less than 24 hours, the inhibiting mechanism was categorized as bacteriostatic inhibition. Pelczar and Chan (1986) stated that the term of bactericidal is used for substances that can kill bacteria and bacteriostatic, it is a state that only inhibits the growth of bacteria so that the bacterial population remained.

3.3. Erythrocyte and Leucocytes Cells

Observation on blood cells of carp (C. carpio) was conducted on the number of erythrocyte and leukocyte. The examined object was observed by using a binocular microscope with a magnification of 400x. Result showed that number of erythrocytes were 224.67x10, 332.67x10, and 417.00x10 cells/mm2 for 730, 750 and 770 ppm Ketapang Bark Crude Extract (KBCE) respectively. In case of leukocytes were 735.89x10, 684.78x10, and 652.67x10 cell/mm2 for 730, 750 and 770 ppm KBCE respectively (Figure 3). According to Monera and Simon (2008), erythrocytes are abundant blood cells in vertebrates. The main function of erythrocytes was for gas exchange (breathing). Structurally, according to Takashi and Hibiya (1995) red blood cells (erythrocytes) in fish have a core, generally it is round and oval depending on the type of fish. Some species had an oval-shaped red blood cells with a diameter of 11-14 µm and had a core with cells volume ratio and the core was 3.5 to 4.0 µm. The number of red blood cells in species were also different. Based on ANOVA test, erythrocytes showed highly significant differences between treatments ($p<0.01$).

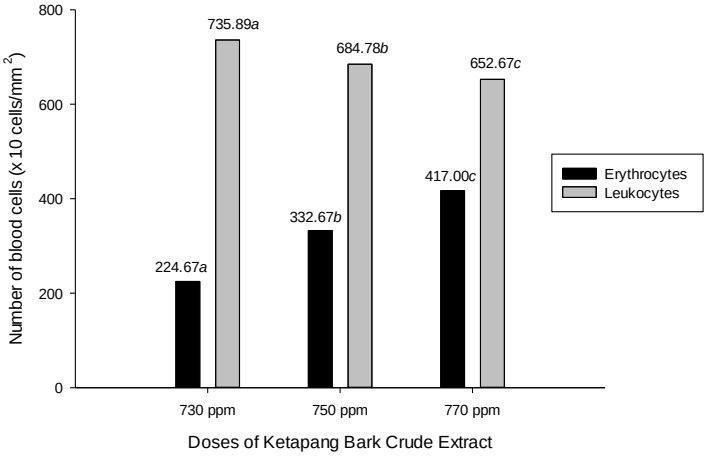

Fig. 3: The Relationship between doses of Ketapang bark crude extract and number of blood cells on Erythrocytes and Leucocytes. Data are represented as mean (n = 9). Different letters indicate significant differences (P<0.01) among three treatments determined by one-way ANOVA and continued by LSD 5%.

Figure 3 showed that the total average of erythrocytes and the use of dose was directly proportional; the higher the dose of the extract given, the more erythrocyte in carp. This indicated that the active ingredient in the ketapang bark crude extract (kbce) was able to effectively inhibit or kill the a. hydrophila which infected the carps. Then, it decreased the bacteria so the inflammation process could be immediately stopped. The impact of the inflammation stopped was indicated by the increasing number of erythrocytes and then leukocytes decrease. This statement was in line with volk and wheeler (1984), phenolic compounds and their derivatives has anti-bacterial characteristic which kill bacterial cells.

According to Kapoor and Khanna (2004), there are two types of white blood cells found in the fish, eosinophils and granulocytes; each of them was one type of neutrophil, granulocyte, lymphocyte, monocyte, and thrombocyte. Leukocytes were the important component in the immune system of fish. The leukocytes production would be directed towards the infected areas as the form of fish defense mechanism. Based on ANOVA test, leukocyte cells indicated significant differences among treatments ($p < 0.01$). The relationship between the different doses and the total erythrocytes could be seen in Figure 3.

The Figure 3 showed that the total average of leukocytes and the dose was inversely proportional; it indicated that the higher dose of KBCE reduced the number of leukocytes in Carp. It could be explained as a mechanism of inflammation decrease as a form of the cessation of infection caused by bacteria or A. hydrophila due to inhibition of bacteria by giving KBCE that contained anti-bacterial active ingredient. In line with the statement from Suhermanto et al., (2011), the rise in the number of leukocytes is an indicator of an infection which can cause inflammation, reversely the decline was due to the expiration of the infection process.

In other cases, Maftuch et al., (2012) and Maftuch et al., (2013) stated that the decrease in blood cells on the shrimp (which is the same function as leukocytes in fish) is because the physiological condition of shrimp which is no longer able to produce haemocytes. In this condition, the shrimp was already in a state of chronic infection as a result of the infection. Meanwhile, in this study the fish was not in the state of chronic infection, it was proven with the continuous increase of erythrocyte cell production as shown with dose and time increase. It could be concluded that the reduction in leukocyte cell was due to inflammation decrease in the body of the carp, as the effect of the cessation of the infection due to death of infecting bacteria by giving of KBCE. This evidence would be explored more in histopathological explanations after the use of the extract.

Water quality parameters during the research was on the level of quality standards that temperature, pH and DO ranged from 24.0 to 25.9°C, from 7.0 to 7.7 and from 3.9 to 4.8 mg/L respectively (Bhatnagar and Devi, 2013).

4. Conclusions and Suggestion

Based on in vitro results, it can be concluded that ketapang bark crude extract with different doses had bacteriostatic characteristic. The biggest diameter of inhibition zone was produced by 1,050 ppm dose with 13.89 mm inhibition zone, and 750 ppm dose produced the lowest inhibition zone with 8.97 mm. In case of in vivo experiment, Ketapang bark crude extract with 770 ppm dose was possible to cure post infection of *A. hydrophila* because it could increase the total of erythrocytes together with the decrease of leucocytes (inflammatory cells) in carp (*C. carpio*). For further research, the histopathology test of carp (*C. carpio*) is expected to be conducted in order to know the histopathology level of recovery after cure.

Acknowledgement

The Authors would like to thank to Gede Agus Andika Kresna Putra and Mirsa Ferdian Putra as a part of research team. We also thank to Heny Suprastyani and Ellana Sanoesi for their valuable advice and suggestion in this research.

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