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Absolute Growth and Survival Rate of Koi Fish Infected with *Aeromonas hydrophila* bacteria with the Treatment of Aloe vera Extract

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ABSTRACT

One of the alternative medicines to overcome *Motile Aeromonas Septicemia* is aloe vera extract. This research aims to study the ability of aloe vera extract to treat koi fish that are attacked by *A. hydrophilla* bacteria. The method used in this research is an experimental method using a completely randomized design (CRD) with five treatments and three replications. The treatment used was dipping koi fish infected with the bacteria *Aeromonas hydrophila* in aloe vera extract with concentrations A (0 ppm), B (150 ppm), C (300 ppm), D (450 ppm) and E (600 ppm).). The parameters observed were survival rates and absolute growth. The results showed that the concentration of 300 ppm aloe extract was effective in treating koi fish infected with *A. hydrophilla* through 48 hours of dipping. It can be seen from the results of the observation of the survival rate and the highest absolute growth of 66.67% and 3.27 g, respectively.

1. INTRODUCTION

One of the highly prospective ornamental fish and technology that is easily mastered by farmers is koi fish. Exporters sell koi to Germany, Africa, Britain, Singapore, and America. The obstacle faced by exporters is to meet the demands of consumers who want the availability of quality koi fish[1], while the obstacles faced by farmers are the attack of Motile Aeromonas Septicemia (MAS) or red spot disease caused by bacterium *Aeromonas hydrophila*. *Aeromonas hydrophila* is one of the diseases that often attack freshwater fish, one of which is koi, especially in the seed stages. *Aeromonas hydrophila* bacteria greatly influence freshwater fish farming and often causes outbreaks of disease with high mortality rates (80-100%) at various sizes in a short period time (1-2 weeks)[2].

Aeromonas hydrophila is an opportunistic type of pathogenic bacteria and can cause systemic diseases and result in mass death[3]. *Aeromonas hydrophila* is a gram-negative, rod-shaped bacterium that can move (motile) and does not form spores. These bacteria are facultatively anaerobic, fermentative, positive cytochrome oxidase, positive catalase and produce H_2S[4]. The clinical symptom of fish affected by *Aeromonas hydrophila* is inflammation with a characteristic swelling at the injection site[5]. The symptoms continue with hemorraghic (bleeding) which is characterized by bleeding on the skin, flaky fins and slow swimming, exophthalmia (swollen eyes), ulcers (and necrosis is a symptom characterized by visible broken and decaying flesh.

Disease prevention in aquaculture systems generally uses antibiotics. However, the use of antibiotics can cause a resistant effect on pathogenic bacteria and can cause pollution in the environment[6]. Therefore alternative drugs are needed that are more eco-friendly and do not cause a resistant effect on bacteria, namely aloe vera extract.

All extracted parts of aloe contain polysaccharides (acemannan & glucomannan), flavonoids, tannins and saponins[7]. The two main polysaccharides contained in aloe vera are glucomannan and acemannan. Glucomannan acts to replace skin tissue and reduce pain due to injury. Acemannan can accelerate wound healing[8]. Flavonoids are anti-inflammatory so they can reduce inflammation and help reduce pain if there is bleeding or inflammation in the wound[9]. Tanin is useful as an antiseptic and also for the treatment of burns by multiplying proteins[10]. The mechanism of saponin as an antibacterial is to damage the constituent components of peptidoglycan on the bacterial cell wall (lipoprotein). This means that the lipid surface tension decreases, the cell permeability changes, and the mechanism can cause bacterial cell function to be abnormal, and the cell lysis and die bacteria[11].

The right way to treat koi fish that is attacked by MAS is by dipping. Treatment with dipping system is the most applicable method compared to injection and feeding because it can simplify the treatment process, especially for fish in large scale sizes [12].

Koi Fish

Koi is included in the cyprinidae group which has an elongated body shape and is slightly compressed as well as the location of its mouth terminal, there are also a pair of tentacles near the mouth. According to[13] the morphology of koi fish is not much different from other types of fish. The body of koi is covered by two layers of skin, namely the outer skin (epidermis) and the inner skin (dermis). The epidermis is useful for protecting the skin from the outside environment, such as from dirt and pests or diseases. The dermis contains pigments or colors such as xantophora (yellow), melanofora (black), guanofora (shiny white), and erythrofora (red)

Koi has a sense of smell, which is a whistle near its mouth that functions to smell food that is in the bottom of muddy waters. With these turtles, koi fish can separate their food from mud. The side there is a linear lateral which is located from the middle of the body to the base of the tail which serves to hear sound vibrations. This line comes from the veins that are next to the fish scales.

Koi fish like a living place (habitat) in fresh waters where the water is not too deep and the flow is not too heavy, such as on the banks of rivers or lakes. Koi fish can live well in areas with an altitude of 150-600 meters above sea level (asl) and at temperatures of 25-30 ° C. Koi fish are classified as omnivores, ie organisms that can prey on various types of food, both from plants and microscopic animals. The main foods are plants and animals found on the bottom and edge of the waters[13].

Aeromonas hydrophila

Aeromonas hydrophila is an opportunistic type of pathogenic bacteria and can cause systemic diseases and result in mass death[2]. This bacterium is often endemic in Southeast Asia and can attack freshwater fish, both ornamental and consumption fish[14]. *Aeromonas hydrophila* is a gram negative, rod shaped, size (0.8 - 1.0) x (1.0 - 3.5) µm. These bacteria can move (motile) and do not form spores. These bacteria are facultative anaerobic, fermentative, positive cytochrome oxidase, positive catalase, produce H_2S, and react positively to the Voges Proskaeur test, indole, hydrolysis of gelatin and arginine dehydrolase. However, these bacteria act negatively on ornitine decarboxylase[4].

This bacterial attack is latent so it does not show symptoms of the disease at first. This disease is only seen in fish after the fish's body resistance decreases due to stress caused by a decrease in water quality, lack of feed or bad handling of fish. Transmission of this disease can be through water, body contact, contact with equipment that has been contaminated or contracted diseases from newly moved fish[15].

Aloe Vera

Aloe vera is included in the Liliaceae family. This plant can grow in tropical and subtropical climates characterized by leaves like knives with sharp jagged edges. Aloe vera leaves have a main component, yellow latex on the outer skin and inner gel (mucilage) [16]. number of studies have reported the benefits of Aloe vera components on health including antimicrobial, anti-inflammatory,

immunomodulatory and antioxidant[17]. All parts of extracted aloe contain polysaccharides (acemanan & glucomannan), flavonoids, tannins and saponins[18].

Useful properties of aloe vera, such as bioactive polysaccharides, specifically storage polymers known as acemannan, and different phenolic compounds, appear to be key components to explain most of the pharmacological properties associated with aloe vera plants[19]. Aloe vera is able to stimulate the immune system. This is because aloe contains acemannan active compounds that are able to activate immune cells[20]. Aloe vera also contains saponin which functions as an antiseptic, besides that quinone compounds in aloe vera are used as antibacterial. Alkaloid compounds in aloe vera are able to increase endurance[21]. Aloe vera also contains tannin compounds as antibacterial. Tanin has antibacterial power by multiplying protein. In general, the antibacterial effects of tannins include reactions with cell membranes, enzyme inactivation and inactivation of the function of bacterial genetic material[22].

Aloe vera polysaccharides have also been linked to direct bacterial activity through stimulation of phagocytic leukocytes, which destroy bacteria[23]. All parts of aloe vera extracted contain polysaccharides and flavonoids. Flavonoids have the potential to be antibacterial, anti-cancer compounds and antibiotics[18]. Flavonoid compounds are synthesized by plants as a defense system and in response to infections by microorganisms, so it is not surprising that these compounds are effective as antimicrobial compounds against a number of microorganisms[24]. Flavonoids are one of the polyphenol compounds that have various effects including antioxidant effects, anti-tumor, anti-inflammatory, antibacterial and anti-viral. Flavonoids work by damaging the cytoplasm so that bacterial cells will be damaged and die. Flavonoids are also anti-inflammatory so they can reduce inflammation and help reduce pain if there is bleeding or inflammation in the wound [8].

Acemannan stimulation of macrophages occurs through mannose receptors found on the surface of macrophage cells. Acemannan increases macrophage activity of the systemic immune system, especially in the blood and spleen and increases the production of macrophage[25]. This is confirmed by the statement of[26] that acemannan polysaccharides have been considered as the main factors of immunomodulatory properties shown by aloe vera for macrophages and monocytes, with minimal systemic toxicity. The healing component is related to a compound called glucomannan, which is enriched with polysaccharides. Glucomannan affects fibroblast growth factors and stimulates cell activity and proliferation and increases collagen production and secretion. Aloe vera mucus not only increases the amount of collagen at the wound site, but also increases the transverse connection between the bonds so as a result it accelerates wound repair[26].

2. MATERIALS AND METHOD

2.1 Time and place of research

This research includes preliminary research and main research. Preliminary research was carried out on March 4-10, 2019 and the main research was carried out for 2 weeks in March and April 2019. This research took place at the Aquaculture Laboratory, Building 4 Fisheries and Agricultural Engineering and Biotechnology Laboratory Building 3 Faculty of Fisheries and Marine Sciences, Padjadjaran University.

2.2 Tools and materials

The tools used are a digital balance, measuring cup, erlenmeyer, rotary evaporator, vial bottle, autoclave, magnetic stirrer, petri dish, L. glass, 6 mm paper disc, bunsen, laminar flow, calipers, aerator, aquarium, thermometer, DO meter, PH meter. The ingredients used are koi fish, aloe vera, methanol, commercial feed (PF 800), A. hydrophilla isolates, and agar media.

2.3 Research Method

The method used in this research is the experimental method using Completely Randomized Design (CRD) with five treatments and three replications. The treatment given is dipping an aloe vera extract on koi fish infected with *Aeromonas hydrophila* with concentration as follows:

- Treatment A : Control treatment without dipping an aloe vera extract
- Treatment B : 150 ppm aloe vera extract
- Treatment C : 300 ppm aloe vera extract
- Treatment D : 450 ppm aloe vera extract
- Treatment E : 600 ppm aloe vera extract

3. RESEARCH PROCEDURE

3.1. Preliminary Research

Extract Making

Making aloe vera extract is done by the maceration method. 30 kg of fresh aloe vera is washed using clean water and dried. After the aloe is dried, then the maceration process is done by dipping the aloe in a methanol solution of 35 L for 3 days. The screening process is then carried out from the results of the maceration process using filter paper to obtain the supernatan. Then the supernatant was evaporated using an evaporator at 80 ° C at a speed of 120x per minute to obtain the extract.

LC_{50} Test

The LC_{50} test was conducted to determine the extraction concentration of aloe vera which caused 50% fish mortality from all

koi fish populations tested for 48 hours using the USEPA method (2002). The LC50 test was repeated twice with a treatment of 10 ppm, 100 ppm, 1000 ppm, and 5000 ppm. First, the test fish is acclimatized for 2 days (48 hours) and given commercial feed twice a day at satiation. Then the fish is put into the aquarium, each 10 fish/aquarium. Each aquarium is given dipping aloe vera extract according to the treatment. Survival rate koi fish data were analyzed using EPA PROBHIT software.

***In Vitro* test**

The *In Vitro* test procedure is carried out in a controlled environment without living organisms in a controlled environment, for example in a test tube or petri dish. The method used in this test is the diffusion method or Kirby-Bauer antibiogram paper disc method.

First, the tools and materials to be used are sterilized by autoclave. Making aloe vera extract by maceration and evaporation, then soaking the paper disc in aloe vera extract according to the concentration of treatment with LC_{50} for 24 hours. Next, make the media. Then the media is poured enough into the petri dish and let stand until dry. A total of 0.5 ml of the culture of *Aeromonas hydrophila* with a density of 10^8 CFU / ml was included in the petri dish containing media, then flattened to the left and right in a balanced manner. Making bacterial solutions of 10^8 CFU / ml. Then put on a paper disk that has been soaked in aloe vera extract in petri dish. Petri dish that has been given a paper disk of aloe vera extract was incubated for 24 hours. Finally, observe the inhibitory zone and measure it using the caliper.

3.2 Main Research

The main studies include preparation of containers, acclimatization of fish, infection with *Aeromonas hydrophila* bacteria, treatment of koi fish with aloe vera extract.

Container Preparation

The preparation of the research container included preparing 15 aquariums and 2 containers. Aquarium and container filled with a ¾ container of water.

Acclimatization of Koi Fish

koi fish are acclimatized for 1 week to adjust fish to new environments.

Koi Fish Infection

Calculated the initial weight of fish biomass when it will be infected with *Aeromonas hydrophila* bacteria. after the fish was acclimatized for 1 week, infected *Aeromonas hydrophila* intramuscularly to koi fish with a density of 10^8 CFU / ml as much as 0.1 ml. After infection *Aeromonas hydrophila* koi fish in aquariums are fed twice a day (8:00 and 16:00) at satiation using commercial feed as much as 3% of body weight per aquarium.

Koi Fish Treatment

Observed clinical symptoms after fish infected with *Aeromonas hydrophila* bacteria, if clinical symptoms appear evenly over the same period time, Aloe vera extract treatment is given according to the treatment for 48 hours. After being treated with aloe vera extract, koi fish are kept for 14 days. Then after 14 days of maintenance, counted dead fish and survivors. As well as calculating the final weight of surviving fish biomass

4. RESULT AND DISCUSSION

4.1 *In Vitro* Test

Table 1. Inhibit Zone Aloe vera extract

Concentration (ppm)	Average inhibitory zone
10	8,39
100	9,77
1000	11,32
5000	20,49

Based on the results of preliminary research on the inhibitory zone test (in vitro) showed that the concentration of aloe vera extract at 5000 ppm had the largest average inhibition zone of 20.49 mm, whereas at a concentration of 10 ppm the average smallest inhibition zone was 8.39. It can be said that Aloe vera extract is a strong antibacterial because it has a diameter of 20 mm inhibition

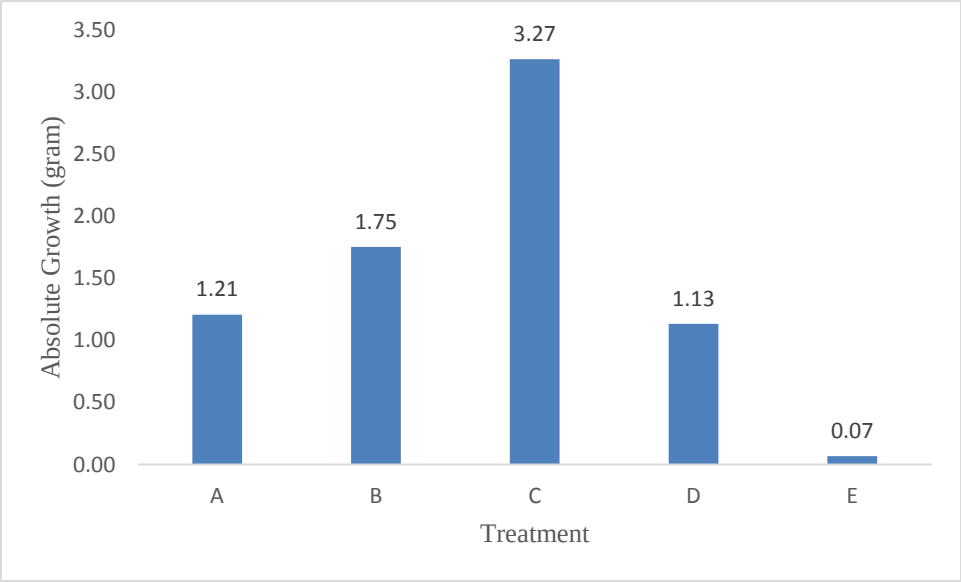
zone.

4.2 *In Vivo* Test

The results of the LC_{50} immersion test 48 hours (in vivo) aloe vera extract on koi fry showed that the concentration of aloe vera extract analyzed using EPA Probit Analysis software found a concentration of 1157.16 ppm could kill fish as much as 50% of total individuals. while the concentration of 304.95 ppm can kill fish as much as 15% of the total individuals.

4.3 Absolute Growth

Based on the results of observations for 14 days the test fish after dipping treatment with aloe vera extract showed absolute growth results that varied in each treatment (Picture 1).



Picture 1. Absolute Growth Graph

Treatment A (control) has an average value of absolute growth (W) of 1.21 g, in treatment B (150 ppm) it experienced an absolute growth to 1.75 g, then increased again to treatment C (300 ppm) with absolute growth 3.27 g, this shows the increasing concentration of treatment with aloe vera extract, the higher the value of growth of absolute weight, but in treatment D (450 ppm) decreased the absolute weight average, this is presumed by the content of saponin in aloe vera extract produce foam that is poisonous and covers aeration, thus inhibiting koi fish breathing. Oxygen levels are an important environmental factor, if the dissolved oxygen concentration is low, the appetite of the cultivated organisms decreases, affecting growth and resistance to disease[27].

Treatment C shows the highest absolute weight growth with an average growth value of absolute weight of 3.27 g, this is caused by the content of antibacterial compounds capable of treating koi fish infected with *Aeromonas hydrophila*, so that in a few days the fish are no longer stressed and fish allocate feed into energy for weight growth [28]. The increase in the weight growth of the average individual indicates that all the feed tested can be used by fish for growth [29]. This is due to the allocation of energy from feed for growth after the energy needs for maintenance are met. While the treatment of E (600 ppm) has the lowest absolute growth value, this is caused by the total death of koi fish on the 1st and 2nd day after treatment due to *Aeromonas hydrophila* infection and toxic saponin content in aloe vera which causes an average the growth rate of individual weight in treatment E only increased from 0 to 0.1 g (Table 2).

Table 2. Absolute Growth Data

Treatment	Average
A (0 ppm)	1,21±0,01a
B (150 ppm)	1,75±0,56a
C (300 ppm)	3,27±0,40a
D (450 ppm)	1,13±0,58a
E (600 ppm)	0,07±0,05a

Note: Values followed by the same letter indicate not significantly different

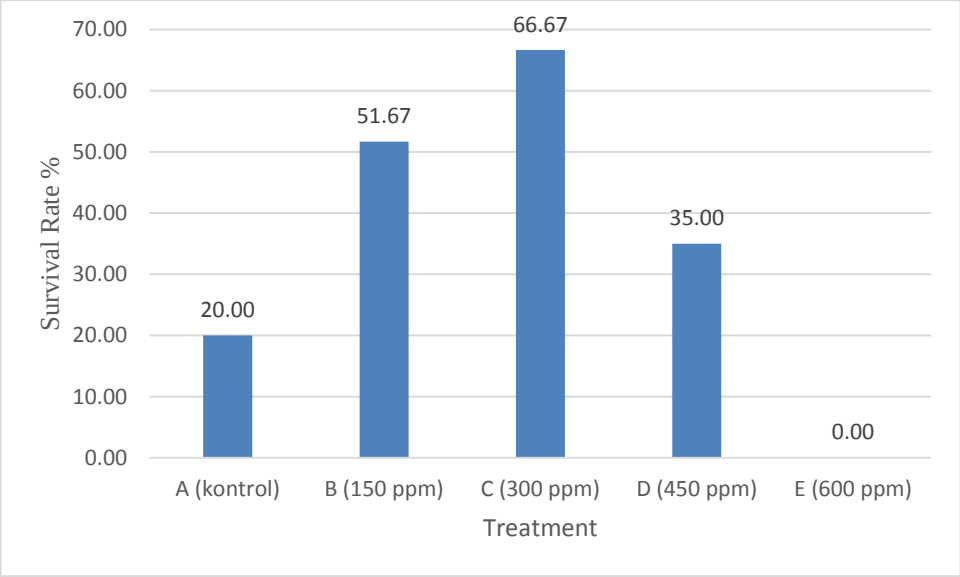
Compared to the research[30] the effect of aloe vera powder on feed on the scented fish tested by A. hydrophilla showed the

highest absolute growth value of 1.77 g. while the absolute growth value of aloe vera extract treatment on koi fish infected with A. hydrophilla with dipping is equal to 3.27 g. This shows that the treatment of aloe extract produces higher weight growth values compared to the prevention of aloe vera powder in feed on ciprinid fish infected with A. hydrophilla..

Based on statistics (Table 2) all treatments of dipping aloe vera extract were not significantly different which meant that dipping aloe vera extract in the treatment of koi fish infected with *Aeromonas hydrophila* bacteria did not effect the growth of weights.

4.4 Survival Rate of Koi Fish

Based on observations for 14 days, it can be concluded that the higher the concentration of aloe vera extract given, the higher the survival rate to a concentration of 300 ppm. At a concentration of 450 ppm, the survival rate decreased and died completely at a concentration of 600 ppm (Picture 2).



Picture 2. The survival rate of koi fish graph

Picture 2 shows that koi fish that were not treated with aloe vera extract (treatment A) showed an average survival rate of 20%. The low value of survival in treatment A (control) is caused by the natural body resistance of koi which is unable to resist the attack of A. hydrophilla and disrupt the metabolism of fish so that it dies. The metabolism of koi is disturbed because the nature of *Aeromonas hydrophila* bacteria can produce exotoxin and endotoxin enzymes that cause hemolysis in the host cell. Exotoxins produced by *Aeromonas hydrophila* such as hemolysin are enzymes that can lyse red blood cells and proteases, namely proteolytic enzymes that function to fight the host's defenses and take stock of host nutrients to multiply [31]. While endotoxin is found in bacterium A, hydrophila in the form of lipopolysaccharide (LPS). LPS can cause inflammation and stress on the host [31].

Treatment B (150 ppm), C (300 ppm), D (450 ppm) and E (600 ppm) showed varying survival. the higher the concentration of aloe extract gives higher survival to certain concentration limits. Treatment B (150 ppm) to treatment C (300 ppm) showed an increase in survival from 51.67% to 66.67%, but with the increasing concentration of the chemical content of aloe vera extract, the higher the saponin content contained therein. This causes a decrease in survival in treatment D (450 ppm) with a value of 35% to total death in treatment E (600 ppm) because of the nature of saponin itself which is toxic to fish when used in high concentrations[32].

According to research[33] the treatment of carp infected with A. hydrophilla using pomegranate peel extract produced the highest survival rate of 57.7%. while the survival rate of the treatment of koi fish infected with A. hydrophilla using aloe vera extract has the highest value of 66.7%. This shows that the treatment using dipping aloe vera extract has a higher survival rate than the treatment of carp using pomegranate peel extract.

The results of variance (Table 3) show that the treatment of aloe vera extract with dipping significantly affected the average survival rate of *Aeromonas hydrophila* koi fish. The Duncan test results at the 95% confidence level showed that the average survival rate of treatment A (control), B (150 ppm), C (300 ppm) and D (450 ppm) was significantly different from treatment E (600 ppm). treatment B (150 ppm) and D (450 ppm) were not significantly different, treatment A (control) was not significantly different from treatment D (450 ppm) but treatment A (control) was significantly different from treatment B (150 ppm). Whereas in treatment C (300 ppm) it was not significantly different from treatment B (150 ppm).

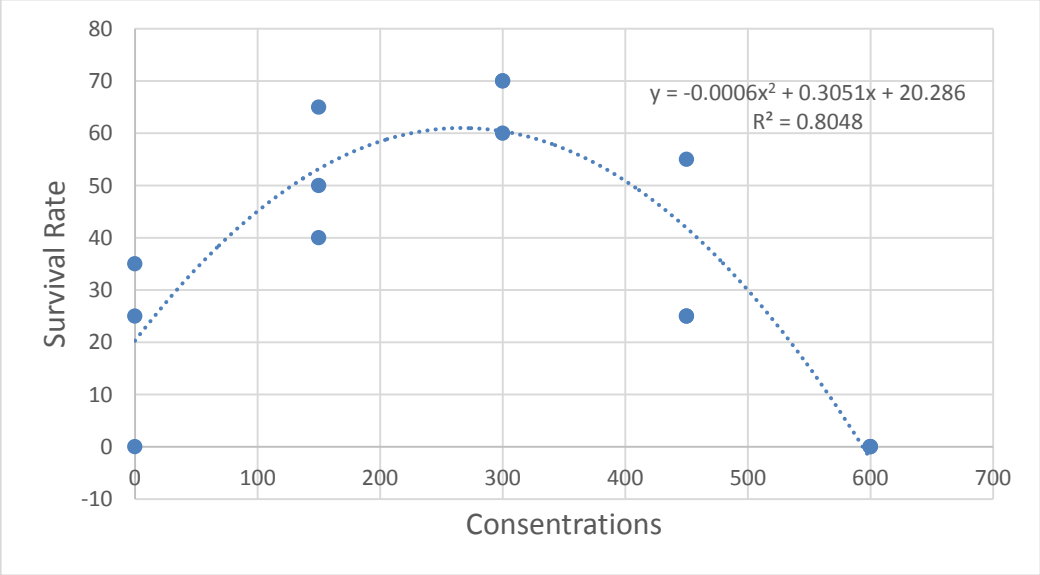
Table 3. Average Survival Rate of Koi Fish

Treatment	Survival Rate %	Arcsin Transformation Results	Notation
A (control)	20,00 ±18.03	22,08	b
B (150 ppm)	51,67 ±12,58	45,97	cd
C (300 ppm)	66,67±5,77	54,76	d
D (450 ppm)	35,00±17,32	35,94	bc
E (600 ppm)	0±0	0,00	a

Note : Values followed by the same letter indicate not significantly different based on Duncan's test at 95% confidence level

Based on Table 3 it can be seen that the koi fish survival rate at treatment E (600 ppm) was the lowest compared to the control treatment and even experienced death to 100%. This happens because the antibacterial compounds contained in aloe vera extract are too high at a concentration of 600 ppm especially toxic saponins. And the natural body resistance of koi is not able to resist the attack of *Aeromonas hydrophila* bacteria. According to the results of the Duncan test treatment E (600 ppm) was significantly different from the other treatments. Treatment B (150 ppm) and D (450 ppm) were not significantly different, treatment B (150 ppm) was significantly different in treatment A (control). but treatment A (control) was not significantly different from treatment D (450 ppm). Whereas in treatment C (300 ppm) it was only significantly different from treatment B (150 ppm).

Treatment B (150 ppm) and treatment C (300 ppm) were not significantly different so there was a need for quadratic regression to determine the optimum concentration between concentrations of 150 ppm - 300 ppm to treat *A. hydrophila* infected koi fish.



Picture 3. Quadratic Regression Survival of Koi Fish

Based on picture 3 shows a regression value of 0.8048 which means the concentration of aloe vera extract affects the survival rate of 80.48% and also found the most optimum concentration of aloe vera extract among concentrations of 150 ppm - 300 ppm which is equal to 266.94 ppm. The optimum results obtained based on quadratic regression showed negative results, this shows that the increasing concentration of aloe vera extract further decreases the survival rate of koi fish.

Aloe vera extract at a concentration of 150 ppm - 300 ppm shows a survival rate above 50%. It states that the antibacterial content in aloe vera extract consisting of flavonoids, tannins, glucomannan and saponins can treat A. hydrophila infection in koi fish.

The mechanism of flavonoids is anti-inflammatory so that it can reduce pain if there is bleeding or swelling[34]. The mechanism of saponin as an antibacterial is to stimulate collagen formation, which is the structure of proteins that play a role in wound healing[35]. The mechanism of the tannin is that it can shrink the cell wall or cell membrane so that permeability is disrupted, the cell cannot carry out living activities so that its growth is inhibited or even dies[36]. While the antibacterial mechanism of glucomannan is replacing skin tissue and reducing pain due to injury[8].

Among all treatments, treatment C (300 ppm) was the most effective concentration in the treatment of koi fish infected with A. hydrrophila because of its higher survival value of 66.67. This shows that treatment C (300 ppm) is the best treatment.

5. CONCLUSION

The results showed that the concentration of 300 ppm aloe extract was effective in treating koi fish infected with A. hydrophilla through 48 hours of dipping. It can be seen from the highest survival rate and absolute growth of 66.67% and 3.27 g, respectively.

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