Use of *Lemna sp* As Antioxidant in Feed and Its Effect on Nile Tilapia (*Oreochromis niloticus*) Performance

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Abstract. This study aimed to understand the antioxidant activities in *Lemna* sp. and its influence on Nile tilapia performance. This study was performed at the Chemical Laboratory and Aquaculture Laboratory in Universitas Padjadjaran during the period of March to July 2018. This was an experimental study using Completely Randomized Design (CRD) with 5 treatments and 4 repetitions. In Treatment A (Feed Without Lemna sp), B (Commercial feed + 25% *Lemna* sp. extract from IC50), C (Commercial feed + 50% *Lemna* sp. extract from IC50), D (Commercial feed + 75% *Lemna* sp. extract from IC50), E (Commercial feed + 100% *Lemna* sp. extract from IC50). Parameters measured were lemna antioxidant activities, daily growth rate, SR, and profile of the Nile tilapia fish. Data collected in this study were analyzed using Analysis of Variance with a confidential level of 95%, followed by Duncan's multiple range test. In vitro extraction was performed on 772 grams of dried lemna with 1,1–diphenyl-2-picryllhydrazil (DPPH) testing using UV-Vis Spectrophotometry. The IC50 value of the mixing dose with commercial feed was then calculated. Ten 9-10 cm Nile tilapia fish was maintained in aquariums for 28 days. The amount of feed provided was 10% of the total biomass of the fish. Results showed that the IC50 value of lemna extract was categorized as quite strong, i.e. 54.517 ppm. The highest daily growth rate, survival rate, and blood cell count was in Treatment B that is 2.36%, 100%, 6.995.000 cells/mm^3 for red blood, and 804.000 cells/mm^3 for leucocytes.

Keywords: antioxidant, blood profile, daily growth rate, *Lemna sp.*, *Oreochromis niloticus*, survival

ICONISTECH 2019, July 11-12, Bandung, Indonesia

DOI 10.4108/eai.11-7-2019.2297619

1 Introduction

Nile tilapia fish is a fresh water fish that has a good prospect to be cultured due to its beneficial biological natures, i.e. easy to culture, fast growing, and has thick flesh [1]. However, fish farming is prone to diseases that often hamper the growth and survival of tilapia fish. These diseases are caused by interactions between fish and unbalanced environment [1]

Fish is very susceptible to reactive oxygen species such as free radicals that can damage the fish tissue. Reactive oxygen species are organic compounds that have a functional group with more oxygen element. To prevent tissue damage, fish must have effective antioxidants for its defense against tissue damage. Vitamin E has the ability to protect tissues from free radical reactions. Fish has the ability to develop antioxidant defenses through the use of its catalase, superoxide dismutase (SOD) antioxidant, glutathione peroxidase, and glutathione reductase enzymes. Together with these enzymes, high-weight molecules such as carotenoids, vitamin E, vitamin K, amino acids, and peptides have been detected in the antioxidant system of fish [2].

Vegetables and fruits are important sources of antioxidants from food while vitamin C, vitamin E, carotenoids, and flavonoids are the primary exogenous antioxidant sources [3]. *Lemna* sp. is considered to contain Vitamin E compounds, carotenoids, and flavonoids that can be used as a source of antioxidants. *Lemna* sp. is a small-sized aquatic plant that floats on water and has the potential to become fresh feed or to be used as feed ingredients because it has a relatively high nutrient content [4].

Flavonoids can function as antioxidants, immunomudulators, and anti-inflammatory agents [5] while some other substances are thought to increase fish immunity, i.e.vaccines, immunostimulants, probiotics, and antioxidants. Active compounds, especially flavonoids, carotenoids and amino acid, of plant extracts including the extract from *Lemna sp.*. are able to play roles in stimulating leukocytes as a non-specific defense, hence functioning as immunostimulants [6]. The aim of this study was to determine antioxidant activities in *Lemna sp.*. as an additional ingredient in the feed given to nile tilapia and to assess the performance of nile tilapia that receives *Lemna* sp. containing feed.

2 Materials and Methodes

Time and place

This study was conducted at the Ciparanje Hatchery, Aquaculture Laboratory, Faculty of Fisheries and Marine Sciences and the Chemistry Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran from March 2018 to July 2018 .

Tools and materials

The tools used in this research are Digital Scale, Rotary Evaporator, Spatula, Filter Paper, Filter Funnel, Vial, Clamp and Support, Ring, Evaporator Flask, Desiccator, Sonicator, Analytical Balance, Micropipette, Measuring Cup, UV-Vis Spectrophotometry, Spray, Analytical Scale, Syringe, Aquarium Small Plate, Mercury Thermometer, Fish Scoop. 0.5 inch Diameter Hose, Aeration Hose and Aeration Rock, Stationery, Millimeter Block, Camera, Zipplock, Potassium Permanganate Solution, Knife, Hemacytometer, Counting Chamber, and Light Microscope. The object of this study were 8-10 cm Nile Tilapia, *Lemna* sp. from Ciparanje Culture Pond, Commercial Feed, Methanol, Hayem's Solution, Turk Solution.

Methods

This was experimental study using the Completely Randomized Design (CRD) of five treatment combinations that were repeated three times. The treatments were:

Treatment A : Feed without *Lemna* sp. (Control).
Treatment B : Commercial feed + 25% *Lemna* sp. extract from IC50
Treatment C : Commercial feed + 50% *Lemna* sp. extract from IC50
Treatment D : Commercial feed + 75% *Lemna* sp. extract from IC50
Treatment E : Commercial feed + 100% *Lemna* sp. extract from IC50

Procedures

Antioxidant Activity Testing

Antioxidant activities of in vitro *Lemna* sp. were initially tested using 1,1 –diphenyl-2-picrylhydrazyl (DPPH). The sample used was 772 grams of dried lemna that was extracted using the maceration method to produce a concentrated extract of 28.21 grams. The antioxidant activities of the Lemna extract were tested using DPPH. This test was performed by adding 1 mL of Lemna extracts in 0, 20, 40, 60, and 80 ppm concentrations into 0.5 mM DPPH solution in a test tube. This mixture was then homogenized with a vortex until it was completely mixed and then allowed to stand in a dark room (away from light and sunlight) with a temperature of about 37℃ for 30 minutes for each sample solution. The absorbance was measured using a UV-Vis spectrophotometers at a wavelength of 514 nm.The antioxidant activities in lemna can be observed from the IC 50 value that was obtained through the % inhibition graph. The IC50 value of the lemna extract is 54,517 ppm. This IC50 value can be used as a reference for adding lemna extract into the fish feed.

Biological Test on Fish

Lemna sp. extract was mixed into fish commercial feed at the doses of 25, 50, 75, and 100% . The formula used to determine the dose of *Lemna* sp. and feed mixture is:

Lemna sp. extract was mixed into fish commercial feed at the doses of 25, 50, 75, and 100% . The formula used to determine the dose of *Lemna* sp. and feed mixture is:

$$\frac{25\%, 50\%, 75\%, 100\%\ lemna\ extract}{1000\ g} x \frac{x}{average\ fish\ weight} \quad (1)$$

Note :
x = lemna extract dose obtained, divided by 7 for weekly treatment

Lemna sp. extract that had been weighed according to the dose used for each treatment was dissolved in 11 ml of NaCl. The solution was homogenized by shaking it in a sprayer. The homogenized solution was then sprayed into the commercial feed. The feed was then aerated to dry.

The test fish was acclimatized for 7 days to allow them to adapt to the new environment. During the adaptation period, the fish was given commercial feed without *Lemna sp.*. On Day 8, the fish started to receive test feed, with an amount of 10% of the fish biomass, three times a day at 08.00, 13.00, and 18.00. The fish density in 14 litres of water was 10 fish. This study was conducted for 28 days with sample weighing performed every 7 days, followed by an adjustment of the amount of feed provided for the following week.

On Day 28, 1 sample fish was collected from each aquarium for blood cell testing. Blood was sampled by putting the fish on a wet cloth to make the blood sampling easier. An incision was then made on the base of the tail until blood oozed out. Blood was then sucked and then Turk and Hayem solutions were homogenized by shaking the thomma pipette. A drop of the produced solution was placed on the hemocitometer and then covered by the cover glass. Observation and calculation were performed using a microscope with 100x magnification.

Parameters observed

1. *Daily Growth Rate*

$$\text{DGR} = \frac{lnWt - lnWo}{t} \times 100\% \quad (2)$$

Notes :
DGR = Daily growth rate (%)
Wt = Average weight of test fish at the end of study (gr)
Wo = Average weight of test fish at the beginning of study (gr)
t = Duration of study/days

2. Survival Rate

$$\text{SR} = \frac{\text{N}_t}{N_0} \times 100\% \quad (3)$$

Notes :

SR : Fish survival rate (%)

N_t : Number of fish at the end of study/fish

N_0 : Number of fish at the beginning of study/fish

3. Blood Profile

a. Red Blood Cell Count

$$\sum \text{RBC per mL blood} = \text{Average number of red blood cells} \times (200 \text{ x } 10 \text{ x } 25) \quad (4)$$

Notes :
200 = Dilution
10 = Hematocytometer thickness
25 = Number of boxes in hemocytometer
Source: [7]

b. White Blood Cell Count

$$\sum \text{WBC per mL blood} = \text{Average number of white blood cells} \times (20 \text{ x } 16 \text{ x } 10) \quad (5)$$

Notes :
20 = Dilution
10 = Hematocytometer thickness
16 = Number of boxes in hemocytometer
Source: [7]

3 Result And Discussion

1. *Daily Growth Rate*

The results of the study on the effect of *Lemna sp.*. addition on the daily growth rate of nile tilapia based on Formula are listed in Table 1 [8].

Table 1. Nile Tilapia Daily Growth Rate

Treatment	Average (%)
A (Commercial feed 100%)	1.34 ± 0.13^{a}
B (Commercial feed 10% + 25% IC50)	2.36 ± 0.57^{c}
C (Commercial feed 10% + 50% IC50)	2.03 ± 0.41^{bc}
D (Commercial feed 10% + 75% IqC_{50})	1.67 ± 0.19^{ab}
E (Commercial feed 10% + 100% IC_{50})	1.69 ± 0.48^{ab}

The highest daily growth rate according to the results of 28 days of observation was seen in treatment B, which was significantly different from those of treatment A (control), treatment D, and treatment E but not significantly different from treatment C. The increase in daily growth rate that was seen in treatment B was considered as showing that the nile tilapia started to be able to absorb nutrition in *Lemna sp.*. that was used as a feed additive. Feed additive is an addition to feed that has a purpose to improve the feed quality. The provision of feed additive may, in addition to increase the growth performance, stimulate the immunity system to increase the metabolism process that will improve the quality of the fish flesh [9]. A feed additive in the form of antioxidant can be used to accelerate the fish growth [10].

In addition to vitamin C, *Lemna* sp. also contains another antioxidant, i.e. vitamin E. Vitamin E is one of the important micronutrients in fish feed as it plays an important role in the growth process, reproduction, health or immune system, as well as in the quality of fish flesh. Vitamin E also functions to maintain intracellular balance and also as antioxidant. The basic need for vitamen E in fish is various, depending on several factors such as the size of the fish, temperature of water, percentage of growth, and composition of feed. Furthermore, it is stated that as an antioxidant, vitamin E can protect fat or fatty acid in the cell membrane from being oxidized [11][12]. In GIFT strain of Nile tilapia (O. niloticus), a diet prepared using corn oil and slightly higher total lipid content of 7.36% increased the vitamin E requirement to 40 mg α-toc/kg diet [13].

The increased growth rate is suspected to be caused by the antioxidant content in *Lemna sp* that is able to be absorbed and consumed well by the fish. The main factor that plays the role in the increased growth rate is because *Lemna sp.* contains antioxidant and vitamin C that can increase growth and is useful for fish normal physiological growth. Vitamin C is able to improve digestion and metabolism in its role as an antioxidant [12].

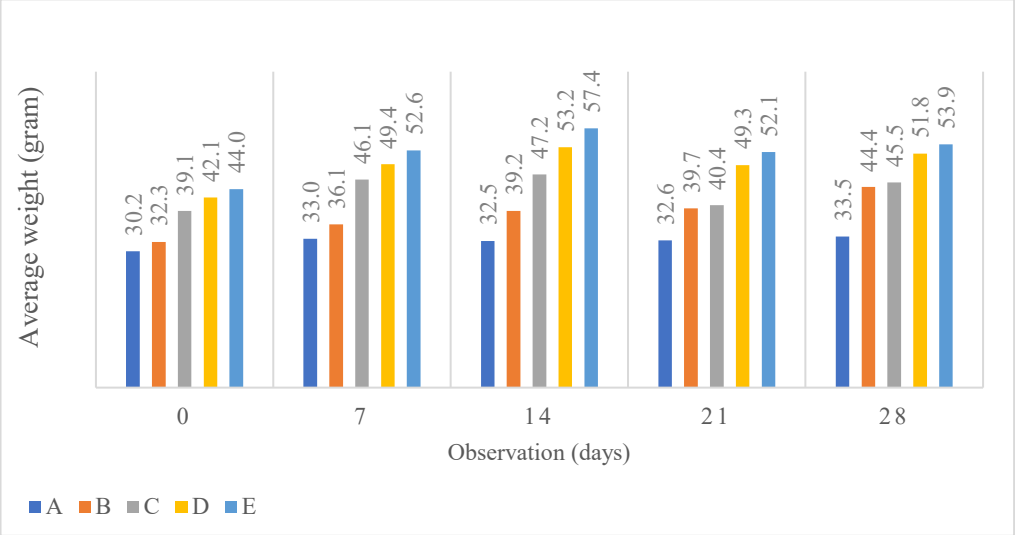

Fig. 1. Fish Weekly Weight Gained

The growth rate of nile tilapia fed by *Lemna sp.*. extract is better than the growth rate in a study on the effect of pineapple extract mix in feed on the growth of 3-5 cm common carp (*Cyprinus carpio*) [13]. The highest growth rate in his study was found in treatment D with addition of 2.25% of pineapple extract, producing a growth rate of 0.84%,

A study on the addition of cinnamon extract in feed and its effects on the growth of 8 cm silver catfish (*Pangasianodon hypophthalmus*) has presented a highest daily growth rate of 2.3% using the dose of 2 grams of cinnamon extract [14]. It is susspected that the hampering activities of cinnamon extract is a bit less than those of lemna extract. Another factor that influence the lower daily growth rate of cinnamon extract is that the dose that is much higher compared to the dose for lemna extract. The anti-nutrition substance in cinnamon leaf extract may hinder the absorption of nutrients that are essential for growth, leading to reduced growth [15]. Another reason for these lower daily growth rates is that cinnamon extract and pinapple extract are thought to play less role as feed additive. Feed additive in the form of antioxidant can be used to improve fish growth performance [10].

2. Blood Profiles

a. Red Blood Cells

Results of the average red blood cell count in the beginning and at the end of the study based on [7] method are described below.

Table 2. Nile tilapia red blood cells

Treatment	Sample Day- (cells/mm^3)				
	0	**7**	**14**	**21**	**28**
A(control)	1380000^a	1577500^a	2080000^a	2812500^a	3197500^a
B	1470000^a	1800000^b	2922500^{ab}	4100000^a	5592500^d
C	1360000^a	2435000^b	3452500^{ab}	4067500^a	2205003^a
D	1510000^a	2325000^b	3352500^b	4482500^d	2395003^c
E	1380000^a	2502500^c	4927500^b	6047500^b	6995000^b

Note: Notation with the same letter in each column represents insignificant difference based on the result of the Duncan's Test with a confidence level of 95%.

Results of the variance analysis on the observation of the number of the red blood cells in nile tilapia before the provision of *Lemna sp.*. extract showed that the number of red blood cells in 1,380,000 cells/mm3 in treatment A, 1,470,000 cells/mm3 in treatment B, 1,360,000 cells/mm3 in treatment C, 1,510,000 cells/mm^3 in treatment D, and 1,380,000 cells/mm^3 in treatment E.

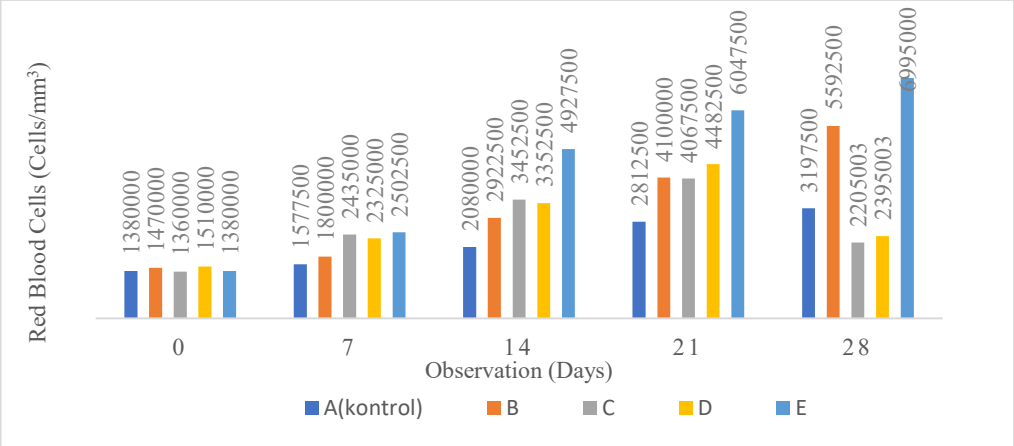

Fig. 2. Increase in Number of Red Blood Cells during Study Period

Results of observation on sample on Day 7 showed significantly difference results when compared to control in each treatment. Provision of *Lemna* sp. Extract seemed to be able to increase the number of red blood cells in each treatment. The treatment with the highest number of red blood cells on Day 7 of the study was treatment E with 2,502,500 cells/mm^3. Observation on sample on Day 4 also gave significantly different results when compared to control based on variance analysis. The highest number of red blood cells was found in treatment E of 4,927,500 cells/mm^3, which was significantly different from the number in treatment C of 3,452,500 cells/mm^3, treatment D of 3,352,500 cells/mm^3, treatment B of 2,922,500 cells/mm^3 and treatment A (Control) of 2,080,000 cells/mm^3.

Sampling on Day 21 did not show significant differences when analyzed using variance analysis. However, an increase was found in each treatment. The highest number of red blood cells was found in treatment E of 6,047,500 cells/mm^3, followed by treatment D of 4,482,500

cells/mm^3, treatment B of 4,100,000 cells/mm^3, and treatment C of 4,067,500 cells/mm^3. A sharp increase was seen on Day 21 for each treatment. On Day 28, the results were not significantly different in each treatment based on the results of the variance analysis. The highest number of red blood cells was seen in treatement E of 6,995,000 cells/mm^3, followed by treatment B of 5,592,500 cells/mm^3, and treatemt A of 3,197,500 cells/mm^3. There was a decrease in the number of red blood into 2,205,003 cells/mm3 cells in treatment C and into 2,395,003 cells/mm^3 in treatment D.

These reductions in the number of red blood cells are suspected to be caused by the anti-nutrition content of lemna in the feed, i.e. saponin. The low erythrocyte count in fish is suggested to be affected by saponin compound because saponin has the ability to lyse red blood cells. The low total erythrocyte count is assumed to be caused by the lysis of red blood cells due to the presence of saponin, leading to reduced total erythrocyte count in fish [16]. The lyzed erythrocytes experience damages in the membrane and hemoglobin that reduce the hemoglobin level. Various sources of stress, including environmental factors (temperature, light, culture, capture, and transport) and biotic factors such as microorganism infection, will negatively affect the physiological changes in animals. Stress may affect physiological activities and hemoglobin level in fish. Blood physiological level varies a lot, depending on the environmental condition such as humidity, temperature, and pH [17].

A study on the effects of the addition of carrot extract on the immunity of comet fish revealed that the most suitable concentration of carrot powder is 0.9% for the initial treatment of 15 days, 1.2% for the treatment of 30 days, and 0.6% for treatment C with the highest number of red blood cells of 5,133,333 cells/ mm^3 [18].

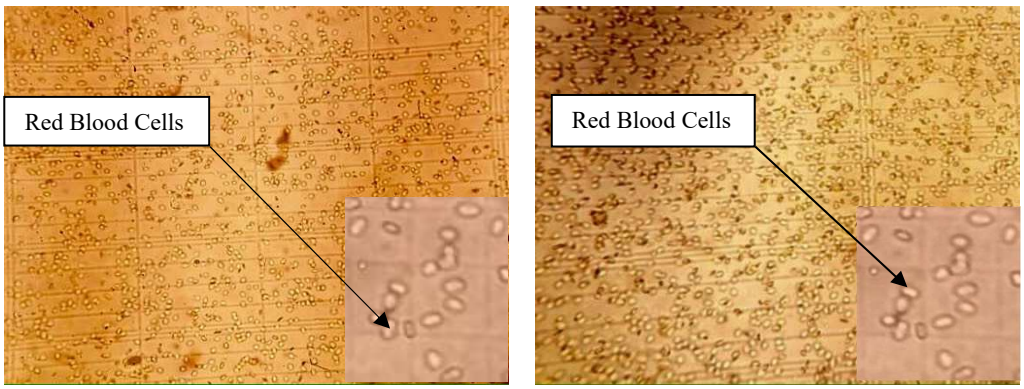

Fig. 3. Comparison of the number of red blood cells before and after the provision of *Lemna sp.*. Extract (Left: before; Right: after)

Antioxidant plays a role in protecting cells (including non-specific immune cells and red blood cells) from the oxidative damages caused by environmental stress. Therefore, fish that is cultured with the addition of antioxidant-containing *Lemna* sp. extract will have better immune response and have blood with better ability to distribute nutrients. The increase in the number of erythrocytes is caused by the presence of flavonoid in *Lemna* sp. extract that functions as an antioxidant [19]. The mechanism of antioxidant in preventing diseases is by increasing the number of erythrocytes to prevent the reduction of the number of erythrocytes during a disease attack in fish.

b. White blood cells

The initial average white blood cell count and the average white blood cell count at the end of the study were calculated using [7] Klontz method (1994). Each week, the number of white blood cells in the Nile tilapia fish experienced changes as described in Table 3.

Table 3. Number of white blood cells in nile tilapia fish (cells/mm^3)

Treatment	Sample Day-				
	0	**7**	**14**	**21**	**28**
A (control)	125300^{a}	141200^{a}	177800^{a}	234000^{a}	283800^{a}
B	134800^{a}	218800^{ab}	334200^{b}	391200^{b}	491600^{ab}
C	136000^{a}	217400^{ab}	325600^{b}	477000^{bd}	545800^{ab}
D	133000^{a}	275000^{b}	400800^{b}	511800^{cd}	665200^{b}
E	130000^{a}	315000^{b}	426200^{b}	591400^{d}	804000^{b}

Note: Notation with the same letter in each column represents insignificant difference based on the result of the Duncan's Test with a confidence level of 95%.

Based on the results of the analysis of variance, the observation on samples on day 7 showed significant differences when compared to the control. Treatment E showed the most significant result of 315,000 cells/mm^3, followed by treatment D of 275,000 cells/mm^3, treatment B of 218,800 cells/mm^3, treatment C of 217,400 cells/mm3, and the lowest was found in treatment A (Control) of 141,200 cells/mm^3.

Observation on Day 14 showed significant differences when compared to the control. The highest number of white blood cells was seen in treatment E of 426,200 cells/mm3, followed by treatment D of 400,800 cells/mm^3, treatment B of 334,200 cells/mm^3, treatment C of 325,600 cells/mm^3, and treatment A (Control) of 177,800 cells/mm^3. On sampling on Day 14, the number of white blood cells was already abundant. Various factors may influence the number of leucocytes in fish, including the condition and health of the fish.

Observation on Day 21 showed significant differences when compared to control, based on the results of the analysis of variance. The highest number of white blood cells was found in treatment E of 591,400 cells/ mm^3, followed by treatment D of 511,800 cells/ mm^3, treatment C of 477,000 cells/ mm^3,treatment B of 391200 cells/ mm3, and, the lowest, control treatment of 234,000 cells/ mm^3.

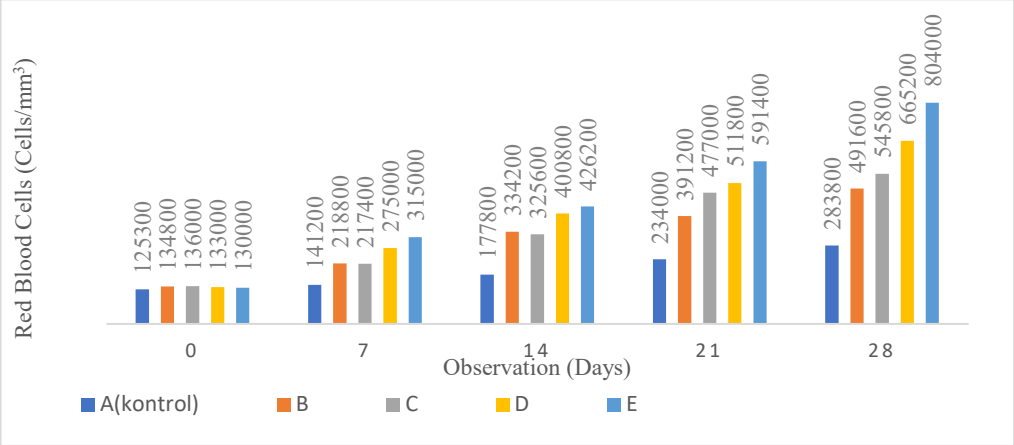

Fig. 4. Increase in Number of White Blood Cells during Study Period

Observation results on Day 14 showed significant differences when compared to the control treatment. The highest number of white blood cells was seen in treatment E of 426,200 cells/mm 3, followed by treatment D of 400,800 cells/mm^3, treatment B of 334,200 cells/mm ^{3}, treatment C of 325,600 cells/mm^3, and treatment A (Control) of 177,800 cells/mm^3. On Day 14, the sample showed that the number of white blood cells were already abundant. Various factors may influence the number of leucocytes in fish, including the condition and health of the fish.

Observations on Day 21 showed significant differences when compared to the control treatment based on the results of the analysis of variance. The highest number of white blood cells was found in treatment E of 591,400 cells/mm^3, followed by treatment D of 511,800 cells/mm3, treatment C of 477,000 cells/mm^3, treatment B of 391,200 cells/mm^3, and control treatment of 234,000cells/mm^3.

Observations on Day 28 showed significant differences when compared to the control treatment based on the results of the analysis of variance. The highest number of white blood cells was found in treatment E of 804,000 cells/mm^3, followed by treatment D of 665,200 cells/mm3, treatment C of 545,800 cells/mm^3, treatment B of 491,000 cells/mm^3, and, the lowest, treatment A (Control) of 283,800 cells/mm^3 .

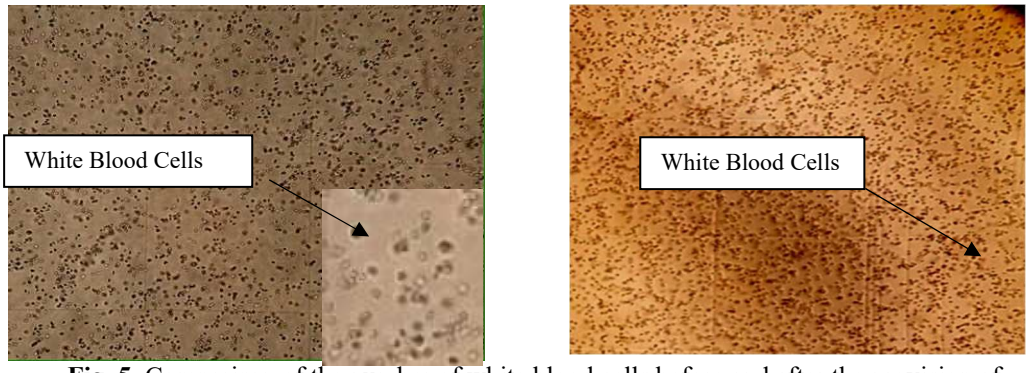

Fig. 5. Comparison of the number of white blood cells before and after the provision of *Lemna* sp. Extract (Left: before; Right: after)

A study by [19] on the effects of the addition of carrot extract on the immunity of comet fish reveals a high white blood cell count of 504,800 cells/mm3, which is still lower than the results gained from adding *Lemna* sp. extract in which the highest white blood cell count is 804,000 cells/mm3. On average, the addition of *Lemna* sp. extract shows significant results for each treatment. However, the most significant result with the most rapid increase was seen in treatment E with the addition of *Lemna* sp. 100% of the IC50 value. This shows that the addition of lemna extract at a dose of 100% from the value of IC50 gives the best result and can increase and influence the number of white blood cells in the fish. Although the control treatment has shown that the fish did not experience a shortage of white blood cells, the higher number of white blood cells may indicate that the fish is able to survive against various sources of disease because it has stronger immunity.

3. Survival

The fish survival rates as the results of the addition of *Lemna sp.*. extract are shown in Table 4 below.

Table 4. Nile Tilapia Survival Rate

Treatment	Average Survival Rate(%)
A 25% IC_{50}	85^{a}
B 50% IC_{50}	100^{b}
C 75% IC_{50}	100^{b}
D 80% IC_{50}	100^{b}
E 100% IC_{50}	100^{b}

Based on the results of the analysis of variance, the addition of lemna extract to the feed in treatment B, treatment C, treatment D, and treatment E results in significant differences when compared to treatment A (Control). Nile tilapia that received lemna extract (with a concentration of 25%, 50%, 75%, and 100%) the survival rate is 100%. The high survival rate is thought to be due to the antioxidant content of lemna that can increase appetite and immunity of the fish. The presence of flavonoids in the antioxidant increases the appetite and immunity to disease and can be used to prevent stress in fish during transportation [20][21].

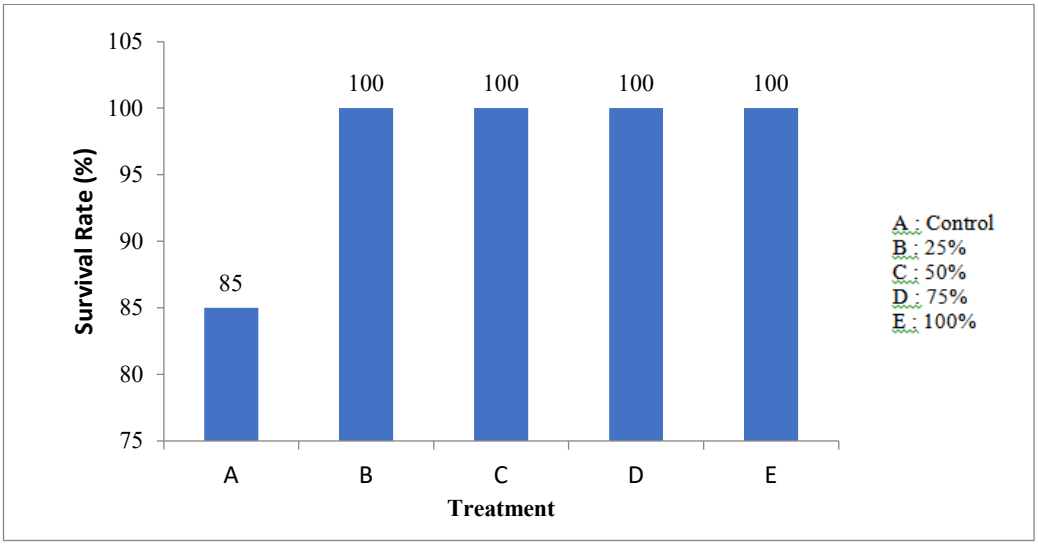

Fig. 6. Survival Rate of Nile Tilapia

The important factor that influences the growth and survival of fish is feeding. The content of free strong anti- radicals in lemna can inhibit the oxidation process, making the fish more immune to disease and survive longer.

A study on the addition of pineapple extract in feed revealed that the survival rate of 3-5 cm common carp (*Cyprinus carpio*) is higher than 90%, although it is still lower than the survival rate seen among fish provided with lemna extract containing feed [13][22]. This is considered to be the effect from the lower antioxidant content in pineapple. There is also a possibility that the fish experienced stress due to inadvertent treatment so that the mortality was high. Food competition may also play a role in this lower survival rate.

A study on the addition of cinnamon extract to feed showed that the survival rate of 8 cm silver catfish (*Pangasianodon hypophthalmus*) is 100%, which is not significantly different from the control [14]. This is presumably because feed that contains cinnamon leaf extract has an effect on the survival of catfish.

4 Conclusion

The provision of 25% IC50 ; lemna extract into commercial feed produces the highest growth rate of 2.36 % with a survival rate of 100% The same dose of *Lemna* sp. extract also increases the immunity of fish, which is characterized by an increased number of red blood cells up to a maximum of 6.995 million cells/mm 3 and the number of white blood cells up to a maximum of 804,000 cells/mm3.

5 Recommendation

It is recommended that further studies should be performed to measure the activities of phytochemicals and antibacteria and that this study should be tested on different fish species.

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