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The Effect of Plant-based Insecticides Contains Active Eugenol and Azadirachtin on Immune System of Common Carp (*Cyprinus carpio* L.)

Ayi Yustiati [a*], Syifa Masyitoh [a], Roffi Grandiosa [a], Titin Herawati [a], Ichsan Nurul Bari [b] and Ibnu Bangkit Bioshina Suryadi [a]

[a] Department of Fisheries, Faculty of Fisheries and Marine Science, Universitas Padjadjaran, Jl. Raya Bandung-Sumedang, Hegarmanah Jatinangor, Sumedang 45363, West Java, Indonesia.
[b] Department of Pest and Plant Disease, Faculty of Agriculture, Universitas Padjadjaran, Jl. Raya Bandung-Sumedang, Hegarmanah Jatinangor, Sumedang 45363, West Java, Indonesia.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

This study aims to investigate the toxicity effects of plant-based insecticides containing *eugenol* and *azadirachtin* on the immune system of Common Carp (*Cyprinus carpio* L.). Several immunological parameters, including white blood cell count, red blood cell count, stress response, and macroscopic clinical symptoms, were examined [1]. The study was conducted from September to October 2022 at the Ciparanje Inland Fisheries Hatchery, Faculty of Fisheries and Marine Sciences, Universitas Padjadjaran. This study employed a completely randomized design with six

**Corresponding author: Email: yustiati@yahoo.com;*

treatments and three replications. The observation period encompassed the measurement of red and white blood cell counts, stress response, and macroscopic clinical symptoms for the initial 14 days. Leukocyte and erythrocyte counts were assessed before and after insecticide exposure on days 3, 7, 10, and 14. Data analysis involved the utilization of quantitative descriptive analysis for red and white blood cell counts. Exposure to plant-based insecticides containing *eugenol* and *azadirachtin* resulted in a suppression of the immune system in common carp fry. However, the insecticide treatment with a concentration of 64 ppm (Treatment F) remained safe for common carp. The highest count of white blood cells was observed in Treatment F (64 ppm) on day 3, while the lowest count was recorded in Treatment A (control). Treatment A (control) also exhibited the highest count of red blood cells, whereas Treatment F (64 ppm) displayed the lowest count. Thus, plant-based insecticides with a concentration of 64 ppm can still be safely utilized in common carp.

Keywords: Azadirachtin; common carp; eugenol; immune system; plant-based insecticides; toxicity effects.

1. INTRODUCTION

Pesticides are chemical substances used to control pests, weeds, fungi, and disease-causing organisms [2]. Based on the research conducted by Purnomo et al. [3], there are three main categories of pesticides: insecticides used to control insects, herbicides used to manage weeds, and fungicides employed to combat fungal diseases in plants. When pesticides are sprayed on crops, a significant portion of them will eventually reach the soil and become residues, which can contaminate the environment. Pesticide residues can have detrimental effects on aquatic ecosystems and can be toxic to aquatic organisms. The direct impacts include the accumulation of pesticides within the body organs due to the consumption of contaminated food or respiratory organ damage, which can temporarily kill cultured fish, reduce disease resistance, and hinder growth [4]. The utilization of conventional insecticides containing synthetic chemicals has become a common practice in pest control within agriculture. However, concerns regarding their negative impacts on human health, the environment, and non-target organisms have heightened the interest in seeking safer and more environmentally friendly alternatives to insecticides [5]. One compelling alternative is the utilization of plant-based insecticides, which harness natural compounds present in plants to control pests. The use of plant-based pesticides offers notable advantages, including the presence of biodegradable residues that undergo rapid degradation, thereby minimizing their environmental persistence. Consequently, these characteristics render plant-based pesticides safe for human and livestock exposure, while mitigating potential adverse impacts on the environment [6]. One commonly used combination of active ingredients in plant-based insecticides is *eugenol* and *azadirachtin.*

Eugenol is a phenolic compound found in essential oils [7], Meanwhile, *azadirachtin* is one of the bioactive compounds found in the neem tree (*Azadirachta indica*). [8]. Both compounds have demonstrated efficacy in controlling diverse plant pests. While plant-based insecticides are regarded as safer alternatives, further research is imperative to evaluate their toxicity effects on non-target organisms, including fish.

Common carp (*Cyprinus carpi*o L.) is one of the freshwater fish species that has high economic value in fish farming [9]. The immune system of fish plays a crucial role in protecting them from infections and diseases [10]. Disruptions in the immune system of fish can lead to a decline in their health and productivity. Common carp is one of the aquatic organisms that is highly sensitive to environmental changes [11].

Therefore, it is important to understand the toxicity effects of plant-based insecticides containing active ingredients *eugenol* and *azadirachtin* on the immune system of common carp. This study aims to investigate the influence of exposure to plant-based insecticides containing *eugenol* and *azadirachtin* on the immune system of common carp fry (*Cyprinus carpio* L.). According to Yustiati et al., (2019), it was stated that various immunological parameters would be tested. The immunological parameters to be tested in this study were reported to include the count of white blood cells, red blood cells, stress response, and macroscopic clinical symptoms [1,12]. This research is expected to provide a better understanding of the potential toxicity effects of plant-based insecticides on common carp, as well as insights for the development of safer and environmentally friendly insecticides [13].

2. MATERIALS AND METHODS

The research was conducted from September to October 2022 at the Ciparanje Inland Fisheries Hatchery, Faculty of Fisheries and Marine Sciences, Universitas Padjadjaran. The preliminary test was carried out in June 2022 at the Aquaculture Laboratory, Faculty of Fisheries and Marine Sciences, Universitas Padjadjaran, and the advanced preliminary test in August at the Ciparanje Inland Fisheries Hatchery, Faculty of Fisheries and Marine Sciences, Universitas Padjadjaran.

This study employed a variety of relevant tools and materials, including fish maintenance equipment, blood sampling apparatus, and environmental parameter measurement devices. The materials utilized comprised fish fry, commercial pellets, plant-based pesticides, and specific solutions. The Common carp fry used as the test subjects were acquired from the Fish Hatchery (BBI) Cibiru in Bandung, West Java. The selected fish were fry measuring 7-8 cm in length, weighing approximately 7-8 g, with a total of 680 individuals. Among them, 120 individuals were allocated for preliminary testing, while 360 individuals were designated as the primary test fish, and 200 individuals were retained as stock.

The research methodology employed was an experimental approach utilizing a completely randomized design (CRD), involving six treatments and three repetitions. The treatments were determined based on preliminary testing and probit analysis [14].

2.1 Preparation of Plant-Based Insecticide Materials

In this study, the plant-based insecticide used contained 20 g/l of *eugenol* and 0.02 g/l of *azadirachtin*. The measurement of the insecticide was carried out using different tools, namely a micropipette with a capacity of 200 µL and tips of 10 µL, as well as a syringe with a capacity of 1 mL and a precision of 0.1 mL. For the concentrations of the insecticide at 12.8 ppm, 25.6 ppm, and 38.4 ppm, the measurements were conducted using the micropipette and transferred into Eppendorf tubes. Meanwhile, the insecticide with concentrations of 51.2 ppm and 64 ppm was measured using a syringe and transferred into cup clip containers.

2.2 Preliminary Test

The preliminary test aimed to determine the range limits of the critical concentration of the test substance for determining the LC50-96 hour, which represents the highest concentration at which the test organisms exhibit no mortality, and the upper threshold concentration, which signifies the lowest concentration causing 100% mortality. In this experiment, treatments were conducted using four dilutions of the plant-based pesticide and one control. The US EPA recommends pollutant concentrations, such as 1, 10, 100, and 1000 ppm [15]. The experiment was replicated twice, and observations were made over a 96-hour period. If the upper threshold and lower threshold values are excessively high, additional testing is performed. The additional testing involved concentrations of 500 ppm, 250 ppm, 175 ppm, 150 ppm, 125 ppm, 100 ppm, 75 ppm, and 50 ppm. The results obtained from the preliminary test provided temporary values for the $LC_{50\text{-}96\ hour}$ concentration.

2.3 Test and Treatmens

Over a duration of 28 days, a testing experiment was conducted, involving the introduction of 20 Common carp fry and a water volume of 40 L into each aquarium. Subsequently, the designated treatments of insecticide were administered. A total of 18 aquariums were utilized in the main trial, each measuring 60 x 29.5 x 35.5 cm^3. Throughout the research period, the fish were provided with a feed equivalent to 4% of their body weight. Feeding occurred thrice daily at 08:00, 12:00, and 16:00 hours (GMT). Additionally, siphoning was carried out in the late afternoon once every three days, removing 10% of the maintenance media volume. During the initial 14 days, measurements were taken for red blood cells, white blood cells, shock response, and macroscopic clinical symptoms.

2.4 Observation of Blood Cells

Observations of white blood cells and red blood cells were performed five times throughout the study. The observations took place prior to the application of the plant-based pesticide treatment and subsequent to the challenge with the plant-based pesticide on day 3, day 7, day 10, and day 14.

2.4.1 Leukocyte collection and analyses

The procedure for obtaining leukocytes [16] involved preparing the microscope and counting chamber, dissecting the test fish, collecting blood using a Thoma pipette, diluting the blood with a Turk's solution, shaking the pipette, dropping the blood fluid into the counting chamber through the Haemacytometer groove, covering it with a cover glass, and counting the white blood cells using a hand counter. The method for calculating the number of leukocytes [16] was determined using the formulation in equation 2.1.

$$WBC = \frac{A1+A2+A3+A4}{4}\ x\ 16\ x\ 10\ x\ 20 \qquad (2.1)$$

Explanation:

WBC = The quantity of leukocytes (white blood cells)
A1+A2+A3+A4 = The quantity of leukocytes in the sample boxes
4 = The quantity of sample boxes
16 = The total number of leukocyte boxes
10 = The thickness of boxes (mm)
20 = The volume of the dilution (mm^3).

2.4.2 Erythrocyte collection and analyses

The procedure for collecting erythrocytes [16] involved aspirating erythrocytes using a pipette with a scale of 0.5, then drawing in Hayem's solution up to the scale of 101, followed by gentle shaking to ensure homogeneity. The first drop was discarded, and the subsequent drops were placed into the hemocytometer and covered with a cover glass. The count was performed in 5 small squares. The method for calculating the number of erythrocytes [16] could be determined using the formulation in equation 2.2.

$$RBC = \frac{A1+A2+A3+A4+A5}{4}\ x\ 25\ x\ 10\ x\ 200 \qquad (2.2)$$

Explanation:

RBC = The quantity of erythrocytes (red blood cells)
A1+A2+A3+A4+A5 = The quantity of erythrocytes in the sample boxes
5 = The quantity of sample boxes
25 = The total number of erythrocytes boxes
10 = The thickness of boxes (mm)
200 = The volume of the dilution (mm^3).

2.5 Data Analysis

The observations regarding leukocytes and erythrocytes were subjected to quantitative descriptive analysis. On the other hand, data pertaining to feeding response, shock response, and macroscopic clinical symptoms were analyzed descriptively in a qualitative manner. The water quality data underwent quantitative descriptive analysis and were compared against the standards specified in the Indonesian National Standard (SNI).

3. RESULTS AND DISCUSSION

3.1 Preliminary Test

Preliminary testing is conducted to obtain the concentration of plant-based insecticide that will be used in the toxicity test. The toxicity test is conducted to determine the effects of pollutants on water. The first step in conducting a toxicity test is to use the lethal concentration 50 (LC_{50}). The lethal concentration 50 (LC_{50}) is a calculation to determine the concentration of a substance or compound within a certain time frame that can cause the death of 50% of a population. The following is a table of the results from the preliminary testing conducted over 96 hours to determine the lower and upper threshold limits.

Table 1. Preliminary test

Concentration (ppm)	Quantity of test fish	Mortality/ 96 hour	Survival Rate (%)
0	30	0	100%
10	30	0	100%
50	30	0	100%
75	30	5	83%
100	30	6	80%
125	30	10	67%
150	30	16	47%
175	30	30	0%
250	30	30	0%
500	30	30	0%
1000	30	30	0%

Table 2. The leukocyte count in the five test calculations

Day to-	Quantity of Leukocytes ($x10^4$) sel/mm^3					
	A (control)	B (12.8ppm)	C (25.6ppm)	D (38.4ppm)	E (51.2ppm)	F (64ppm)
0	6.63±0.27a	6.63±0.27a	6.63±0.27a	6.63±0.27a	6.63±0.27a	6.63±0.27a
3	6.56±0.24a	7.02±0.37bc	8.17±0.38cd	9.22±0.07de	9.74±0.02ef	10.26±0.15f
7	6.68±0.18a	6.86±0.36a	7.80±0.23b	8.75±0.35cd	9.35±0.15de	9.75±0.27e
10	6.60±0.31a	6.73±0.50a	7.58±0.19b	8.54±0.34cd	8.92±0.36d	9.24±0.59d
14	6.54±0.32a	6.58±0.33a	6.95±0.22a	7.8±0.08b	8.41±0.13cd	8.81±0.04d

The results of the preliminary testing conducted on the toxicity of plant-based insecticides containing eugenol and azadirachtin on common carp fingerlings as non-target organisms indicate that the test fish experienced 100% mortality at a concentration of 175 ppm over a 24-hour period, while at a concentration of 50 ppm, all test fish remained alive for 94 hours. From the fish mortality data in the preliminary test, it can be observed that the concentration of 175 ppm serves as the upper threshold limit, and the concentration of 50 ppm serves as the lower threshold limit. Subsequently, the mortality values were analyzed using probit regression analysis with the SPSS 2021 program to determine the value of the LC_{50}. The probit regression analysis yielded an LC_{50} value of 128 ppm for the plant-based insecticides containing eugenol and azadirachtin.

3.2 Leukocytes

Leukocytes serve as the body's immune defense system against foreign entities. Hence, leukocyte measurement is conducted to ascertain variations in leukocyte count among different treatments in Common carp. The calculation is performed five times, namely prior to the application of plant-based insecticide treatment and following plant-based insecticide challenge on day 3, day 7, day 10, and day 14. The data illustrating the examination of leukocyte count parameter for several concentrations of natural insecticide can be elucidated in Table 1.

Based on the data presented in Table 1, the observed results of white blood cell analysis have been subjected to ANOVA. These data indicate the calculated white blood cell count of Common carp fry on day 0, or prior to any treatment, with an average count of 6.63×10^4 cells/mm3. This count signifies that the Common carp fry were in a normal condition. The leukocyte count for Common carp was reported to be within the range of $2.73\text{-}3.47 \times 10^4$ cells/mm^3 [10], as stated by [17]. Another study by [17] confirmed a white blood cell count of 6.57×10^4 cells/mm^3 for Common carp fry. Similarly, [18] reported a slightly different count of 6.88×10^4 cells/mm^3. However, following the application of varying concentrations of plant-based insecticide, the average white blood cell count of Common carp exhibited an increase ranging from 7.02 to 10.26×10^4 cells/mm^3.

The leukocyte count in Common carp fry treated with plant-based insecticides containing *eugeno*l and *azadirachtin* was found to be higher compared to the control group. This indicates that the fish experiencing an increase in leukocytes are in an unhealthy state. Fish exposed to insecticides exhibit signs of stress, such as erratic movements, staying near the water surface more frequently, and reduced response to feed. An increase in leukocyte count can occur in stressed fish [19]. This response is influenced by corticosteroid hormones and is non-specific, resulting from stressors affected by environmental factors [19]. Stress in fish can disrupt the immune system, which has a negative impact on growth and survival [20].

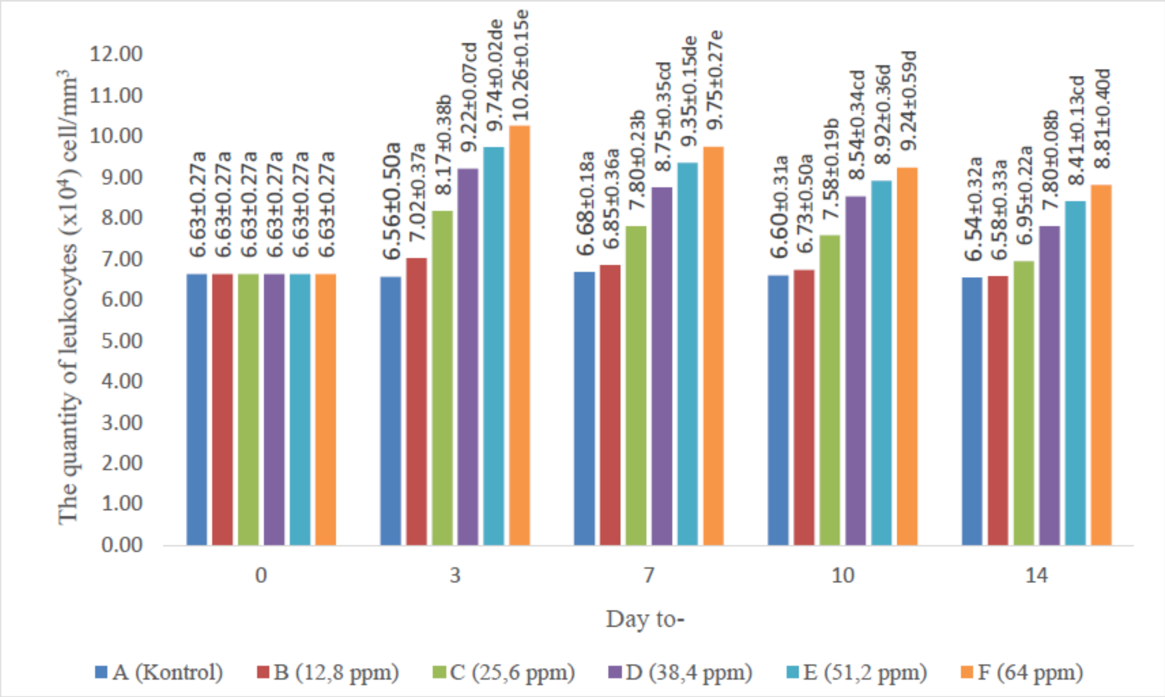

Fig. 1. The graph illustrates the correlation between leukocyte count and observation time

Based on the findings presented in Fig. 1, the white blood cell count exhibits a significant increase on the third day, reaching its peak. Among the treatments, the highest white blood cell count is observed in treatment F (64 ppm), while the lowest count is observed in treatment A (control) at 6.56×10^4 cells/mm^3. The higher white blood cell count compared to the control treatment suggests that the fish are in an infected state [21]. Subsequently, on the seventh day, the white blood cell count begins to decline, indicating a healing phase. This decline in white blood cell count continues until the fourteenth day. Hence, it can be concluded that Common carp fry exposed to plant-based insecticides containing *eugenol* and *azadirachtin* experience a significant increase in the average white blood cell count. Furthermore, there is a positive correlation between the concentration of the plant-based insecticide and the magnitude of the white blood cell count elevation in Common carp fry.

3.3 Erythrocytes

Erythrocytes play a crucial role in the binding and transportation of oxygen from the gills to the entire body of fish, where it is subsequently utilized in catabolic processes to generate energy. Hematocrit measurements are conducted to ascertain the changes in erythrocyte count when exposed to plant-based pesticides containing active ingredients *eugenol* and *azadirachtin*. The calculations are performed five times, namely before the phytochemical treatment and after challenging with the pesticide on days 3, 7, 10, and 14. The data obtained from testing the parameter of red blood cell count for various concentrations of natural insecticides are outlined in Table 2.

Table 3. The erythrocytes count in the five test calculations

Day to-	Quantity of erythrocytes (x10^6) cell/mm^3					
	A (Control)	B (12.8ppm)	C (25.6ppm)	D (38.4ppm)	E (51.2ppm)	F (64ppm)
0	1.60±0.06a	1.60±0.06a	1.60±0.06a	1.60±0.06a	1.60±0.06a	1.60±0.06a
3	1.65±0.05a	1.3±0.09b	1.19±0.03cd	0.99±0.05d	0.77±0.05de	0.63±0.12e
7	1.68±0.03a	1.41±0.08a	1.28±0.11b	1.15±0.13cd	0.89±0.10de	0.78±0.5e
10	1.61±0.13a	1.51±0.02a	1.36±0.15b	1.22±0.06cd	1.01±0.03de	0.88±0.03e
14	1.66±0.14a	1.62±0.9b	1.44±0.04b	1.33±0.08cd	1.18±0.03de	1.01±0.08e

Based on the data presented in Table 1, the red blood cell count before treatment (day 0) is recorded as 1.6 × 10^4 cells/mm^3. This value falls within the normal range. It is consistent with previous research, which reported the red blood cell count in Common carp fry to be around 1.33 - 1.52 × 10^4 cells/mm^3 [22]. Furthermore, according to the research conducted by Irianto [23], the red blood cell count in bony fish ranges from approximately 1,050,000 to 3,000,000 per mm^3 of blood. The visual representation of this data table is depicted in the graph shown in Fig. 2.

Based on the findings presented in Fig. 2, it is observed that on day 3, the red blood cell count in Common carp fry starts to decrease following the treatment. The highest value is recorded in treatment B (12.8 ppm) at 1.3 × 106 cells/mm3, while the lowest count is found in treatment F (64 ppm) at 0.63 × 106 cells/mm3. This decline indicates that Common carp fry experience disruptions due to the effects of plant-based insecticides. Subsequently, after being treated with plant-based insecticides on days 7, 10, and 14, there is an increase in red blood cells in Common carp fry. By day 14, the average red blood cell count reaches the normal range. This signifies that infected Common carp fry are in the healing phase. The rise in red blood cell count suggests that the fish's body is making efforts to defend itself by generating more red blood cells to replace those that have undergone lysis [12]. Based on the data presented in Fig. 2, it can be observed that as the concentration of the plant-based insecticide administered increases, the red blood cell count in Common carp fry decreases.

3.4 Feeding Response

One effort to assess the health of fish is by observing their response to feed. Healthy fish have a high appetite. When given feed, the fish will immediately respond to the provided feed [23-25].

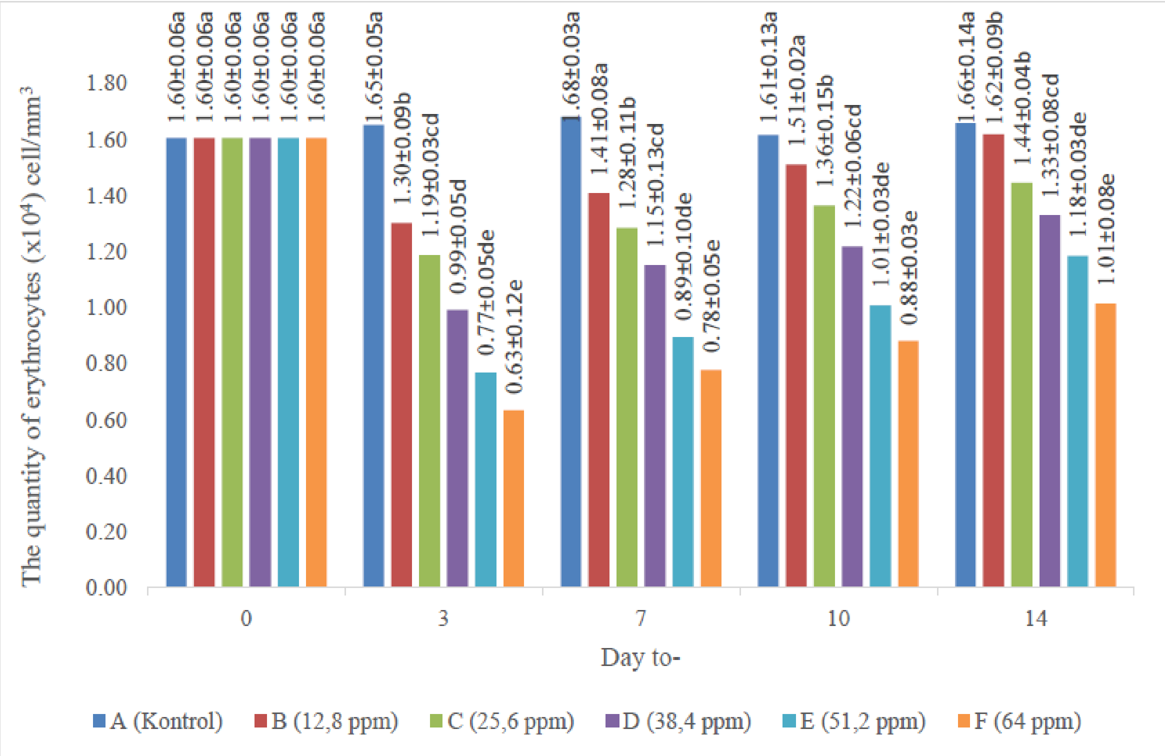

Fig. 2. The graph illustrates the correlation between erythrocytes count and observation time

Table 4. The feeding response in common carp

Treatments	Repetitions	Feeding Response Day to--													
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
A	1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
B	1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
C	1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
D	1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
E	1	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
F	1	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

Explanation: (+++) Immediate response to feed
(++) Response to feed within <3 minutes
(+) Response to feed after >3 minutes
(-) No response to feed

Observations of the response to feed conducted showed that on the first day, fish treated with plant-based insecticides at concentrations D (38.4 ppm), treatment E (51.2 ppm), and treatment F (64 ppm) experienced a decrease in appetite. This is suspected to be caused by toxic substances found in plant-based insecticides that contain active ingredients such as eugenol and azadirachtin. According to the study by [26], it has been found that the decrease in fish appetite is due to the toxic effects of pesticides, specifically herbicides containing the active ingredient isopropylamine. The decrease in fish appetite is a result of stress caused by the toxic substances in plant-based insecticides. Fish contaminated with toxic substances will clinically exhibit signs of stress, such as a decrease in appetite and irregular movements [6]. This is one of the fish's efforts to reduce the biochemical processes in the body that are affected by toxic substances, thereby slowing down the lethal effects. The decrease in appetite in fish exposed to insecticides can also be caused by damage to the gills due to toxic substances from the insecticides [27]. As a result, fish infected by foreign bodies will have disrupted appetite [12], due to the disturbance in their metabolism caused by hypoxia in the body tissues [27].

The feeding response of common carp treated with botanical insecticides containing the active ingredients eugenol and azadirachtin on the third day, particularly in treatments D (38.4 ppm), E (51.2 ppm), and F (64 ppm), returned to normal. This is believed to indicate that the fish exposed to the insecticides are starting to recover and adapt to their environment. According to [24], healthy fish will respond well to feed.

3.5 Shock Response

The response to shock is conducted to evaluate the condition of the fish following the application of plant-based insecticide treatment. The feeding response observation entails tapping the aquarium to observe the fish's reaction to external stimuli [12]. Healthy fish will promptly respond to the shock with rapid movements [21]. The results of the shock response observation in Common carp treated with plant-based insecticides can be seen in Table 3.

Table 5. The shock response in common carp

Treatments	Repetitions	Shock Response Day to--													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
A	1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
B	1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
C	1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
D	1	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
E	1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
F	1	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

Explanation: (+++) responded to shock in less than 30 seconds
(++) responded to shock in 31-90 seconds
(+) responded to shock in 90-180 seconds
(-) did not respond to shock

3.6 Clinical Macroscopic Symptoms

Observations of macroscopic clinical symptoms were conducted after the treatment by examining external body damage such as the fish's body surface, hemorrhagic, and scale shedding. Clinical symptom observations were performed up to day 14.

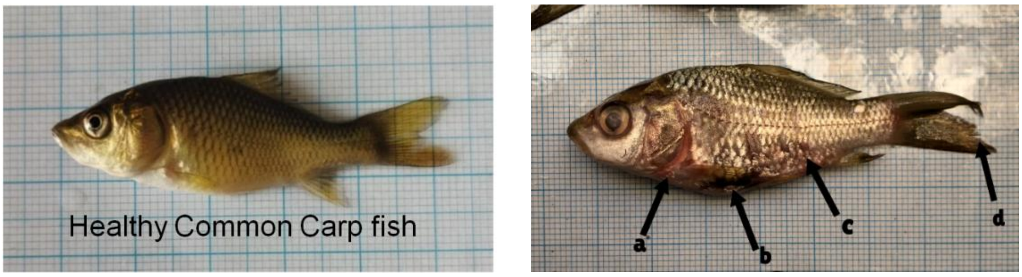

Fig. 3. Clinical symptoms: (a) hemorrhagic, (b) ulcers, (c) scale shedding, (d) frayed fins

Based on the display in Fig. 3, the observation results after the fish were subjected to the treatment show clinical symptoms such as hemorrhage, ulceration, scale shedding, and frayed fins. Hemorrhage is caused by the hemolytic enzymes entering the blood vessels and lysing the red blood cells, resulting in a reddish coloration on the fish's body surface [31]. Ulceration in fish is caused by exposure to the plant-based insecticide, as it contains the active ingredient *eugenol*, which can cause burning effects [7]. Fish treated with the plant-based insecticide experienced stress, characterized by erratic movements, swimming to the water surface, faster gill movement, and excessive mucus production. These symptoms indicate that the fish are responding to protect themselves [23].

4. CONCLUSION

The exposure to plant-based insecticides containing *eugenol* and *azadirachtin* resulted in a decrease in the immune system of common carp juveniles. However, the insecticide treatment with a concentration of 64 ppm (Treatment F) remained safe for common carp fingerling cultivation. White blood cells exhibited the highest count in Treatment F (64 ppm) on day 3, while the lowest count was observed in Treatment A (control). Treatment A (control) also displayed the highest count of red blood cells, whereas Treatment F (64 ppm) had the lowest count. Thus, plant-based insecticides with a concentration of 64 ppm can still be safely utilized in the cultivation of Common Carp.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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