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Analysis of Giving Ethanol Extract of Red Ginger in Feed on Hematology and Differential Leukocytes in Catfish (*Clarias gariepinus*)

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ABSTRACT: Catfish *(Clarias gariepinus)* is an important aquaculture species, especially in Indonesia. The current obstacle faced by farmers is the attack of pathogenic disease outbreaks which have a negative impact on production and cause economic losses. Therefore, this research was designed to evaluate the immunostimulatory effect of red ginger extract in feed on the hematological and immune responses of catfish. Red Ginger is extracted using ethanol. Phytochemical tests show that red ginger extract contains phenolics, alkaloids and flavonoids. Red ginger extract with various concentrations (50, 100, 150, 200 ppm) was mixed with commercial feed and given to catfish for 14 days of rearing. The results showed that there was no significant effect (P>0.05) on the number of erythrocyte cells between treatments. The same thing happened to total leukocytes in catfish, which did not show a significant effect (p>0.05). However, there was a significant effect (P<0.05) on lymphocytes and monocytes, but not on neutrophils (P>0.05). This research recommends using red ginger extract with a concentration of 150 ppm to improve the hematological performance and immune response of catfish. This research provides new insights into the potential of ginger as an immunostimulant agent in aquaculture.

Keywords: Clarias gariepinus, Red Ginger Extract, Immunostimulant, Phytochemical Test

Introduction

One of the aquaculture commodities that has quite a high demand in Indonesia is catfish (Clarias gariepinus) (Wardhana et al., 2021; Araujo et al., 2022), because it has high protein, has high economic value, and can live well. high stocking density, fast growth, and easy to cultivate (Yuliantoro et al., 2017). The obstacle currently faced by farmers is a decline in catfish production caused by various factors, including attacks by fish disease outbreaks (Wise et al., 2021) which are pathogenic in nature, such as parasites, fungi, viruses and bacteria (Korni et al., 2017; Hoa et al., 2021).

Rahma et al., (2015) stated that one of the effective prevention efforts carried out on catfish so that they are not easily attacked by disease is by increasing non-specific immunity by administering

immunostimulants and anti-bacterial/antibiotics (Mondal et al., 2022). Ziyadaturrohmah et al., (2013) added that efforts to prevent and control diseases in catfish, especially those caused by bacteria, currently still use antibiotics or chemicals. Using plants as natural ingredients can be a good alternative to replace the role of chemicals because they do not cause dangerous side effects, are environmentally friendly, do not require large costs and can be easily obtained (Azkiyah, 2020).

One type of plant that can be used to prevent disease is the ginger plant (Zingiber officinale). Ginger has several varieties, one of which is Red Ginger (Zingiber officinale Rubrum) (Pebiningrum and Kusnadi, 2017; Zhang et al., 2022). The compounds contained in ginger plants that are used are the result of secondary metabolite processes such as flavonoids, terpenoids, essential oils and phenols (Alkandahri et al., 2020). These compounds have the potential to act as anti-bacterials and immunostimulants so they can improve fish health (Azkiyah, 2020).

The research results of Indriani et al., (2014) stated that the use of Red Ginger extract (Z. officinale var. Rubrum) as an alternative treatment for Tilapia (O. niloticus). Furthermore, the results of research by Nurjanah and Fathia (2017) found that dried ginger extract has antimicrobial activity against several pathogenic bacteria. And in research by Khuluq et al., (2021), red ginger extract as an immunomodulator can increase phagocytic activity. Blood is one way to diagnose the health status of fish. Blood experiences serious changes, especially when exposed to infectious diseases (Amlacher 1970; Rahma et al., 2015).

Based on this information, this research aims to determine the optimum dose of red ginger extract in feed which affects hematological performance and its effect in improving the immune system of catfish (*Clarias gariepinus*).

Methods

This research was carried out from January to July 2023 at the Laboratory of Fish Parasites and Diseases, Faculty of Marine and Fisheries Sciences, Hasanuddin University and activities for making red ginger extract were carried out at the Water Quality Laboratory, Faculty of Marine and Fisheries Sciences, Hasanuddin University. The tools used are autoclave, Erlenmeyer, centrifuge, petri dish, grinder, knife, test tube, beaker glass, Eppendorf tube, cotton, stick, ose needle, tweezers, disc paper (Whatman no. 42), label paper, incubator, vortex, object glass, cover glass, microscope, dropper pipette, stir bar, micropipette, gauze, spirit lamp, rubber scale, aluminum foil, 10 ml micro pipette, ruler and writing utensils. The ingredients used are Red Ginger Extract, distilled water, 96% alcohol, 70% alcohol, Catfish (Clarias gariepinus), and commercial fish feed.

Research Procedures

Preparation of Container

The containers used in this research were 15 fiber tubs. Before use, the research containers are washed thoroughly using a disinfectant in the form of chlorine. After cleaning, each container is filled with 10 liters of water, then an aeration installation is installed in each container.

Preparation and extraction of ginger plants

3 kilograms of red ginger was obtained from a traditional market in Makassar. Then the ginger is washed using running water until clean and free of adhering dirt and unused rhizomes (rotten, dry, etc.) are discarded. Then it is sliced and dried by drying it out of direct sunlight until dry. The dried red ginger is ground and sifted with a 30 mesh to become simplicia powder and stored in a closed container. Extraction of ginger plants is carried out by maceration using a solvent that can filter out most of the secondary metabolites contained in ginger, namely ethanol solvent. Put one part of dry simplicia powder into the macerator, then add 10 parts of solvent. Soak for the first 6 hours, stirring occasionally, then let sit for 18 hours. Separate the maserate by centrifugation. Repeat the filtration process at least once with the same type of solvent and half the volume of solvent in the first filtration. Collect all the maserate, then evaporate it with a vacuum evaporator or low pressure evaporator until a thick extract is obtained, namely a concentration of 100%.

Preparation of Test Feed

The feed used in this research is commercial feed in the form of pellets which are commonly used in catfish farming. The red ginger extract was diluted according to the concentration required in the research, then 2 mL was taken and then sprayed into 50 grams of feed. The sprayed feed is dried by airing before being given to the fish.

Maintenance of Test Fish

The test fish were kept in a fiber tank for 1 week for the acclimatization process and given pelleted food that had not been added with ginger extract at a dose of 3% of body weight/day with a frequency of 3 (three) times a day. After the acclimatization process, the fish were transferred into a research container equipped with an aerator with a density of 10 fish/container, but beforehand the initial weight was weighed for each of the 3 sample fish. Treatment feeding lasted for 14 days at a dose of 3% of body weight/day with a frequency of 3 (three) times a day. During the treatment feeding period, water quality is maintained in good condition by replacing water and measuring water quality.

Treatment and Experimental Design

This research used a completely randomized design (CRD) consisting of 5 treatments with 3 replications to obtain 15 experimental units.

The treatment in this research is as follows:

A = Red Ginger plant extract with a concentration of 50 ppm + Feed

B = Red Ginger plant extract with a concentration of 100 ppm + Feed

C = Red Ginger plant extract with a concentration of 150 ppm + Feed

D = Red Ginger plant extract with a concentration of 200 ppm + Feed

E = Without giving Red Ginger extract

Research parameters

Phytochemical test of ginger plants

Identification of antibacterial compounds in the ethanol extract of red ginger is through the Phytochemical Test including identification of phenolics, flavonoids, alkaloids, saponins, steroids and triterpenoids.

Total Erythrocytes

The fish observation procedure begins by taking fish blood at the base of the catfish's tail using a 1 ml syringe, then the fish blood that has been taken is placed in an Eppendorf tube that has been filled with EDTA liquid. Then the blood that has been collected is sucked with a thoma pipette (which contains red stirring beads), then Hayem's solution is added to a scale of 101, then the blood in the pipette is homogenized by swinging the hand holding the pipette in a figure eight shape for 3-5 minutes so that the blood is mixed. flat. The first two drops of blood solution in the pipette are discarded, then dripped into the hemocytometer and covered with a cover glass. Then, count the number of red blood cells with the help of a microscope with a magnification of 400 x. The total number of erythrocytes is counted in 5 small boxes and the number is calculated according to the Biaxhall and Daisley (1973) formula, namely :

$$\sum \text{Eritrosit (sel/mm}^3\text{)} = \text{number of calculated cells x 50 x 200}$$

Total Leukocytes

The observation procedure begins by taking fish blood from the base of the catfish's tail using a 1 ml syringe and then collecting it in an Eppendorf that has been given EDTA. Next, take the blood that has been collected using a Thoma Leukocyte pipette up to a scale of 0.5 (then continue by taking Turk's solution up to a scale of 11). The blood sample is then homogenized by shaking the pipette in a figure 8 shape for 3-5 minutes to make it homogeneous. The first drop of blood that has been homogenized is

removed first, while the next drop is put into the hemocytometer and covered using a cover glass. Then carry out calculations on 4 large hemocytometer boxes based on the Blaxhall and Daisley (1973) method, namely :

$$\sum \text{Leukosit (cells/mm}^3) = (\sum \text{counted cells})/(0{,}4 \text{ mm}^3) \times 20$$

Leukocyte Differentiation

The differential leukocytes counted are divided into three, namely lymphocytes, monocytes and neutrophils. According to Arsal et al., (2014) the first step is to soak the glass object in a methanol solution so that the fat and dirt on the glass object will wash off. Then the blood of the catfish (C. Gariepinus) was dripped onto the glass object. Next, another glass object is placed on top of the blood drop until it forms an angle of around 300 then pulled until the blood spreads along the first glass object. The blood was air dried and stained using Giemsa solution for 15 minutes. Then rinse with running water and observe under a microscope. The calculation of the number of lymphocytes, monocytes and neutrophils according to Hartika et al., (2014) is :

$$\% \text{ lymphocytes} = L/100 \text{ X } 100\%$$

$$\% \text{ Monocytes} = M/100 \text{ X } 100\%$$

$$\% \text{ Neutrophils} = N/100 \text{ X } 100\ \%$$

Data analysis

Phytochemical test data were analyzed descriptively and data on hematological performance and immune response were analyzed statistically using analysis of variance (ANOVA), and if the results obtained had a significant effect, continued with the W-Tukey further test.

Results and Discussion

Phytochemical Test

Based on the Phytochemical test screening, the results obtained were that the extraction of Red Ginger with Ethanol as a solvent contained several compounds including flavonoids, alkaloids and tannins/phenols, as presented in Table 1.

Table 1. Phytochemical Test of Red Ginger Extract

No	Secondary metabolites	Red Ginger Extract
1	Phenolic	Positive
2	Flavonoids	Positive
3	Alkaloids	Positive
4	Saponins	Negative
5	Steroids	Negative
6	Triterpenoids	Negative

Source: Hasanuddin University Biochemistry Laboratory, 2023

Based on table 1. Shows the results of phytochemical screening of red ginger extract. Red ginger extract contains secondary metabolites from the phenolic group, alkaloids and flavonoids. This is caused by the broad spectrum of polarity of the ethanol solvent so that it is able to dissolve polar, semi-polar and non-polar compounds (Sasebohe et al., 2023). However, in the phytochemical test of red ginger extract, no saponin, steroid and triterpenoid compounds were found. This is because the phytochemical screening test has advantages and disadvantages. The advantages are that the secondary metabolite testing method is the simplest and fastest, selective in identifying certain compounds, and can provide additional information on the presence of compounds in the samples studied. Meanwhile, the drawback can be known as a false positive reaction, the test results state positive but are actually negative, this is due to equipment error, inaccuracy, and the influence of compounds that have acidic or basic properties (Syafira et al., 2022).

Ramdhini et al., (2022) in their research found that red ginger extract contains flavonoids, alkaloids, saponins and tannins. This compound functions as an antipyretic, analgesic, antifungal, antiseptic, antibacterial, hepatoprotector, antirepplent, anti-expectorant and immunomodulator (Kumalasari and Andiarna, 2020). Another study reported Kela et al., (2023) that phytochemical screening of several medicinal plants revealed the presence of alkaloids, carbohydrates , flavonoids, saponins and phenolic compounds are associated with antimicrobial activity and curative properties against pathogens. Furthermore, research from Herawati and Saptarini (2019) carried out phytochemical screening which aimed to show the secondary metabolite groups found in simplicia from red ginger extract. The results of his research showed that the secondary metabolites detected were alkaloids, flavonoids, tannins, polyphenols, saponins.

Catfish Erythrocytes (C. Gariepinus)

Based on the results of observations on the total erythrocytes of catfish (*C. gariepinus*) which were fed with additional red ginger extract after 14 days of rearing, the erythrocyte values were obtained as presented in Table 2.

Table 2. Results of measuring the number of catfish erythrocytes

Treatment (ppm)	Average Number of Catfish Erythrocyte Cells (10^6 cells/µL)
A (50)	1,10±0,05[a]
B (100)	1,23±0,34[a]
C (150)	3,20±0,96[a]
D (200)	2,98±1,77[a]
E (Control)	0,87±0,09[a]

Note: The same superscript letter in the column indicates there is no difference between treatments at the 95% confidence level (P>0.05).

Based on the data from the ANOVA test results in Table 2, it shows that the treatments have no significant effect (P>0.05). Even though there was no real effect between treatments, the number of erythrocytes in catfish increased with each additional dose of red ginger extract. Descriptively, catfish fed with 150 ppm red ginger extract showed a higher total erythrocyte, namely 3.20x106 cells/µL, this number is still within the normal range. The number of normal African catfish erythrocytes ranges from 2 - 3 x 106 cells/mm^3 (Cerlina et al., 2021). Meanwhile, the lowest total erythrocytes were in controls without administration of red ginger extract, namely 0.87x106 cells/µL. The total erythrocyte value in the treatment given red ginger extract had a higher value compared to the treatment without red ginger extract, this is because red ginger extract can increase erythrocyte cells in fish blood. Ahmed et al., (2023) concluded that ginger is a strong natural antioxidant which, when supplemented with food, can protect red blood cells from hemolysis caused by free radicals, thereby extending the life of fish..

Catfish Leukocytes (*C. gariepinus*)

Based on the results of observations on the total leukocytes of catfish (C. gariepinus) which were given feed with the addition of red ginger extract after 14 days of rearing, the leukocyte values were presented in Table 3.

Table 3. Results of measuring the number of catfish leukocytes

Treatment (ppm)	**Average Number of Catfish Leukocyte Cells (Cells/mm^3)**
A (50)	214.000±23446,74[a]
B (100)	188.333±40565,79[a]
C (150)	179.333±61066,22[a]
D (200)	168.500±52144,03[a]
E (Control)	203.500±20808,65[a]

Note: The same superscript letter in the column indicates there is no difference between treatments at the 95% confidence level (P>0.05).

Based on the data from the analysis of variance (ANOVA) in Table 3, it shows that feeding with different doses of Red Ginger extract did not have a significant effect on the total leukocytes of catfish (P>0.05). The highest total leukocytes were obtained in fish given 50 ppm red ginger extract, namely 214,000 cells/mm^3.

Leukocytes in catfish fed with additional red ginger extract did not have significant differences in each treatment. However, the number of leukocytes in catfish that were given additional red ginger extract in their feed was higher compared to the control. The highest leukocyte value was in treatment with a dose of 50 ppm, namely 214,000 cells/mm. The increase in the number of leukocytes is thought to be due to an increase in the body's defense against bacterial infections. This is confirmed by Ginting et al., (2021) that an increase in total leukocytes in the blood indicates that the fish's immune system has increased, which is characterized by an increase in phagocytic cells which function to carry out phagocytosis of foreign objects that enter the fish's body. The number of leukocytes in fish infected with pathogens will increase as an effort to defend the body (Lestari et al., 2017). Added by Cunha et al., (2019) the phenolic content in red ginger rhizomes is thought to have a role as a regulator of immunity by synthesizing pro-inflammatory cytokines, influencing immune cell regulation and gene expression.

Then the number of leukocytes decreased after the dose of red ginger extract was increased to 100 ppm (treatment B), namely 188,333 cells/m^3, then 150 ppm (treatment C) 179,333 cells/m^3 and the lowest at a dose of 200 ppm, namely 168,500 cells/m^3. A similar thing was reported in research by Riztawati et al., (2023) that total leukocytes that had been soaked using ginger rhizome extract and maintained for 14 days showed a leukocyte yield of 56,004 cells/mm^3, while fish that had not been soaked showed a leukocyte yield of 165,856 cells/mm^3. Nainggolan et al., (2021) explained that the range of leukocyte counts for normal fish is around 150,000-300,000 cells/mm^3. It can be concluded that the number of leukocytes in the blood of catfish in this study was still within normal limits.

Leukocyte Differential

Differential leukocyte observations are used to determine differences in the percentage of leukocyte cell components. The components of leukocyte cells include 3 cells, namely lymphocytes, monocytes and neutrophils. The results of differential observations of leukocytes in catfish (C. gariepinus) after giving Red Ginger extract in feed after 14 days of rearing are presented in tabular form, namely in Table 4.

Table 4. Average Differential Number of Catfish Leukocytes During the Study (%)

Red Ginger Extract	Leukocyte Differential (%)		
	Lymphocytes	Monocytes	Neutrophils
A (50 ppm)	72,7±3,5^{ab}	17,6±1,5^{ab}	14,3±3,5^{a}
B (100 ppm)	78,7±3,2^{ab}	16,3±1,5^{a}	12,6±2,8^{a}
C (150 ppm)	80,3±1,5^{b}	14,3±0,6^{a}	10,3±1,5^{a}
D (200 ppm)	80,0±2,6^{b}	16±1,0^{a}	9,6±2,8^{a}
E (Control)	71,3±3,1^{a}	20,3±1,5^{b}	16,6±2,0^{a}

Note: The same superscript letter in the column indicates a significant difference between treatments at the 95% confidence level (P<0.05).

Based on the results of the analysis of variance (ANOVA), it showed that feeding with the addition of different doses of Red Ginger extract showed a significant effect on the Lymphocytes and Monocytes of Catfish (P<0.05). However, it had no real effect on catfish neutrophils (P>0.05). Based on the research results in table 4. The number of lymphocyte cells in catfish after feeding with additional red ginger extract ranged from 71.7% - 82.3%. The normal percentage of lymphocytes in catfish ranges from 71.12-82.88%. Lymphocytes are the most dominant type of leukocyte cell in the leukocyte population in fish. Through these observations, it is known that Red Ginger extract helps lymphocytes increase in number

(Preanger et al., 2016). An increase in the percentage of lymphocyte cells is a sign of the success of the immune system in developing a cellular (non-specific) immune response (Lestari et al., 2019).

The percentage of monocyte cells found in catfish after adding red ginger extract to feed ranged from 19.3% to 25.3%. Meanwhile, the normal percentage of monocytes in teleost fish is 0.1% of the leukocyte population, but can increase rapidly (around 48 hours) after infection with foreign objects such as carbon (Preanger et al., 2016). The number of monocyte cells decreases as the dose of red ginger extract added to the feed increases. This is thought to be because the activity of monocytes as phagocytic cells is not too high. With the activity of ginger which acts as an antibacterial, the compound components of ginger can prevent the entry of pathogens into the body (Fajryani et al., 2017) added by Ginting et al., (2015) Monocytes which tend to decrease are related to the function of monocytes as macrophages, where monocytes are not needed to phagocytes, because there is no infection that has entered the body which stimulates monocyte production.

The percentage of neutrophils calculated in this study ranged from 9.6% -16.6%. Erman et al., (2021) concluded that the number of neutrophils in normal fish is around 6-8% of the total leukocytes in fish blood. Based on the table, the number of neutrophils continues to decrease along with increasing doses of Red Ginger extract given in the feed. A decrease in the percentage of neutrophils indicates that the fish is in healthy condition and there is no attack by microorganisms. In accordance with the opinion of Hartika et al., (2014), a low percentage of neutrophils indicates that there is no attack by microorganisms so that not many neutrophils are produced by the fish's body.

Apart from that, the active compound contained in red ginger, namely tannin, has an effect on neutrophil cells. The mechanism of action of tannin compounds is that they bind to cell membranes and extracellular proteins in bacteria, resulting in inhibition of the growth of microorganisms (Mufti et al., 2022).

Conclusion

Based on the results of the research that has been carried out, the conclusion is that Red Ginger Extract contains secondary metabolites in the form of flavonoids, phenolics and alkaloids which can act as antibacterials and immunostimulants thereby improving fish health. Providing Red Ginger extract in Catfish (C. gariepinus) feed for 14 days can increase erythrocyte and leukocyte cells as well as the immune response (Differential Leukocytes) of Catfish. A dose of 150 ppm red ginger extract is the best dose because it gives the best results in every parameter.

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