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# Antibacterial Application of a Combination of Moringa and Red Ginger Extracts on the White Snapper *(Lates calcarifer)* Infected with *Vibrio alginolyticus*

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## ABSTRACT

The purpose of this study was to investigate the effectiveness of extracts from *Moringa oleifera* leaves and red ginger (*Zingiber officinale* var. *rubrum*) in inhibiting *Vibrio alginolyticus* bacteria cultured from the white snapper (*Lates calcarifer*). Three extract combinations were tested: A (75% moringa leaves, 25% red ginger), B (50% moringa leaves, 50% red ginger), and C (25% moringa leaves, 75% red ginger), with amoxicillin as a control (treatment K) for the antibacterial activity test. The sizes of the inhibition zones formed after 24 hours were measured to assess the effectiveness of the extracts. Additionally, tests were conducted to observe the responses of fish immersed in the bacterial solution. The healing effectiveness of the extracts on infected fish was documented. This research utilized a completely randomized design with four treatments and three replications focused on fish physiological responses. Results indicated no significant difference among the combinations of *Moringa oleifera* and *Zingiber officinale* var. *rubrum* in inhibiting the growth of *Vibrio alginolyticus* bacteria ($P > 0.05$) *in vitro*. However, *in vivo* tests showed that treatment K (without extract) and treatment A caused the most damage to the fish, including one individual death. Treatments B and C demonstrated signs of recovery, with healing processes beginning after two days of application. Treatment K exhibited the highest lymphocyte value at 86.67%, followed by treatments A, B, and C at 76.67, 75.67, and 74.33%, respectively.

## INTRODUCTION

The bacterium *V. alginolyticus* often causes vibriosis, a disease that can cause mass mortality in fish, particularly in larval and juvenile stages within 1-2 weeks post-infection **(Yanuar *et al.*, 2022)**. This bacterial infection often affects fish species such as the Asian seabass (*L. calcarifer*), cobia (*Rachycentron canadum*), hybrid groupers (Camouflage grouper, *E. polyphekadion*), tiger grouper (*E. fuscoguttatus*) **(Krupesha Sharma *et al.* 2012; Rameshkumar *et al.* 2014; Mohamad *et al.*, 2019)**. This constant infection can kill 90–95% of the snapper population if left untreated due to its high systemic infection impact **(Islam *et al*., 2024)**.

Antibiotics are one method to prevent infection, but their use must be carefully considered to avoid bacterial resistance from uncontrolled use, which could hinder the organism's ability to fight or inhibit pathogens **(Pattah, 2020)**. According to **Okeke *et al*. (2022)**, the use of antibiotics can leave residues in fish bodies, posing a risk to consumers if they consume the fish. One way to reduce the use of antibiotics in aquaculture is to add natural raw materials that contain bioactive molecules and have an impact on fish health and growth **(Hoseinifar *et al*., 2024)**. Moringa leaves extract and red ginger are two natural antibiotic ingredients that can inhibit bacterial activity **(Widyaningsih *et al*., 2022)**.

According to **Oghenochuko and Mshelbwala (2021)**, *Moringa oleifera*, also known as Moringa leaves, contains bioactive chemical compounds such as alkaloids, flavonoids, phenolics, triterpenoids/steroids, and tannins that function as antibacterial and cancer drugs is a type of tropical plant that has grown and developed in Indonesia. Researchers report that the antibacterial compounds in moringa leaves suppress the growth of *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus* **(Rahayu *et al*., 2021; Royani *et al*., 2023)**. **Mahmoud *et al*. (2021)** and **Windarti and Kurniawan (2023)** reported that the addition of moringa leaves in fish feed reduces pathogenic bacteria in the tilapia and catfish. **Abidin *et al*. (2021)** obtained similar information, demonstrating that moringa leaves can inhibit the activity of *Vibrio alginolyticus* bacteria in the *Vanamei* shrimp.

Red ginger is a type of plant that can be used as medicine and a mixture of flavors in food, usually growing in lowland areas, sometimes up to mountainous areas, with an altitude of 0–1500 meters above sea level **(Dhanik *et al*., 2017; Hutabarat *et al*., 2023)**. Red ginger, in addition to its use as a herbal medicine, possesses various groups of secondary metabolic compounds such as alkaloids, flavonoids, phenolics, triterpenoids, and saponins **(Kaban *et al*. 2016)**. In general, the content of compounds produced by Zingiberaceae plants can inhibit the growth of pathogenic bacteria **(Handrianto, 2016)**. Previous research revealed that red ginger extract was able to inhibit *Argulus japonicus* in the goldfish **(Laurensia & Amin, 2023)**.

In the above reviews, this study used a combination of extracts from red ginger and different moringa leaves as an alternative natural antibiotic to overcome diseases caused by *Vibrio alginolyticus* bacteria in the white snapper fish.

## MATERIALS AND METHODS

### Materials and tools

Materials used in this study were moringa leaves and red ginger (Fig. 1), methanol solution as the solvent, nutrient agar, disc paper, filter paper, alcohol, *Vibrio alginolyticus* bacterial isolate, amoxicillin, and sterile distilled water. The tools used were autoclave, petridisc, measuring cup, incubator, vernier, Erlenmeyer, round bottom flask, magnetic stirrer, thermoshaker, micropipette, rotary evaporator, test tube, analytical balance, vial, ose needle, hotplate, freezer, laminar air flow, incubator, blender, wrap paper and aluminum foil.

**Fig. 1.** Moringa leaves (left) and red ginger (right)

**Preparation for materials extraction**

*Moringa oleifera* and *Zingiber officinale* var. *rubrum* were transported to Ambon Island from Luhu Village, West Seram Regency, in a coolbox to maintain their quality of freshness. Moringa leaf and red ginger extracts were prepared in the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Pattimura University. Moringa leaves were separated from their stems and dried in a 50°C oven for one hour. The moringa leaves were then processed into powder using a blender without adding water. The fine moringa leaf powder was dissolved in 600ml of 96% ethanol for 2x24 hours, stirred once a day and kept out of the sun. The maceration product was then filtered using Whatman filter paper to eliminate any residue and evaporated using a rotary evaporator at a temperature of $60^0$C for 15 minutes to separate the solvent from the extract. The end result was a 33-gram paste extract derived from one kilogram of moringa leaves. For the red ginger extract, the same technique was used using thin slices of 5 kilos of red ginger, yielding a 37-gram paste extract.

**Phytochemical screening**

The phytochemical tests of *Moringa oleifera* and red ginger *Zingiber officinale* var. *rubrum* extracts were conducted at the Basic Chemistry Laboratory, Pattimura University. It includes the detection of compounds of flavonoids, alkaloids, steroids/triterpenoids and tannins, while the analysis of saponin and phenol compounds refers to Harborne's research. The methods used for testing include the alkaloid test with NaOH solution, the flavonoid test with concentrated $NH_4OH$, the terpenoid and steroid tests with anhydrous acetic acid, and the tannin test with $FeCl_3$.

**Preparation of test bacteria**

Natrium agar (NA) media was made by dissolving 20 grams of NA powder in 1L of distilled water. The medium was homogenized using a stirrer, heated on a hot plate, and sterilized in an autoclave at 121°C for 15 minutes. Once sterilized, it was poured directly into each petri dish while still liquid at 45–50°C. Vibrio alginolyticus bacteria, obtained as oblique isolates from the Fishery Products Technology Laboratory at the Faculty of Fisheries and Marine Science, Pattimura University, were transferred using a sterile ose needle previously burned with a Bunsen flame to prevent contamination. After the needle was cooled, the bacteria were transferred to culture plates containing NA media and incubated at 37°C for 2 hours.

**Preparation of extract solution**

Three concentrations of extract combinations were prepared for antibacterial testing: 75% moringa leaves and 25% red ginger (A), 50% moringa leaves and 50% red ginger (B), and 25% moringa leaves and 75% red ginger (C).

To prepare a 25% concentration, the following steps were followed:

1. Multiply 25% by 1ml (the required solution volume).
2. Transfer the calculated volume of the extract into a microtube.
3. Add 0.75ml of distilled water.
4. Vortex the mixture to ensure thorough mixing.

The procedure for preparing 50 and 75% concentrations is identical, with the only variation being the amount of extract used. Once prepared, each extract was mixed according to the specified combination design.

**Antibacterial activity testing**

The diameter of the inhibition zone for the test bacteria, *Vibrio alginolyticus*, was measured to conduct the antibacterial test. The first step involved dripping extracts of moringa leaves and red ginger at various concentrations (A, B, and C) using a 50μL micropipette. Antibacterial testing was performed using the disc diffusion method (Kirby-Bauer test).

A sterile disc was inserted into a test tube containing a bacterial suspension and was then applied to NA media. Once the bacterial spread dried, a 6 mm paper disk soaked in the extract for 1 hour was drained and placed on the media containing the bacterial spread, applying slight pressure to ensure the disk adhered to the surface. The plates were then incubated at 37°C for 24 to 48 hours.

If a circular zone of inhibition forms around the paper disk, it indicates positive antibacterial activity. For the negative control, distilled water was used as the solution, which did not show any antibacterial activity. The positive control involved using commercial antibiotics to measure the diameter of the inhibition zone formed.

**Exposure of bacteria on fish samples and lymphocytes count**

36 fish specimens used in the study were maintained in 12 Styrofoam boxes, equipped with an aeration system, with 20 liters of seawater and were acclimatized. The fish were reared for 5 days fed with pellet twice a day until satiation. Observation of fish covered 15 days, 3 days after the bacterial infection, 5 days of infection process, and 7 days of healing process, following the immersion with combination of extract with different concentration. Sustaining appropriate feeding and water quality conditions was

conducted to keep the fish from becoming stressed. Continuous observation was used to assess behavior **(Fanggidae *et al*., 2018)**.

The density of bacteria used refers to **Setyowati *et al*. (2014)** and **Qomariyah *et al*. (2017)**. Bacterial exposure was done in 1L of solution, containing of 5ml of $10^8$ CFU/ml bacterial solution and 995ml of seawater for 10 minutes. After that, the fish were transferred into the rearing container and observed for clinical signs occurred for 24 hours. Fish infected with fish bacteria showed symptoms such as: (1) Loss of appetite, (2) blackened body color such as burning/body color fading, (4) loss of balance, (5) abnormal/slow swimming, and (6) excessive mucus discharge **(Novriadi *et al*., 2014)**.

Observation of white blood cell types was done with Giemsa staining **(Hartika *et al*., 2014)**. The dried blood preparations were observed under a microscope with 400x magnification. The percentage of lymphocyte cells was calculated by observing 10 fields of view, and each type of lymphocyte was differentiated and counted. The calculation of lymphocyte cells in percentage is (Lymphocytes (%) = L/100 x 100%, where L = the number of lymphocyte cells in white blood cells.

**Data analysis**

This study used a completely randomized design (CRD) with four treatments and three replications. The parameters tested statistically were the inhibition zone measurement parameters. The obtained data were tabulated using the MS Office Excel 2013 program, and testing for the normality and homogeneity of the data was performed before conducting the ANOVA test using the SPSS 24.0. Differences between treatments were determined by testing results using analysis of variance with a 95% confidence interval. If the F test yielded significantly different results, data were then proceeded with the Duncan test.

## RESULTS

### 1. Screening test

Screening test results showed moringa leaves extract containing complete active compounds, namely alkaloids, flavonoids, phenols, terpenoids, steroids, and tannins. Based on phytochemical tests conducted by **Bagheri *et al*. (2020)**, moringa leaves contain chemical compounds such as alkaloids, tannins, phenolic compounds, terpenoids, carbohydrates, amino acids, and oils that act as anti-bacterial and anti-microbial.

**Table 1.** Phytochemical test results of moringa leaves and red ginger extracts

| No | Bioactive compound | Color | Result | |
|---|---|---|---|---|
| | | | *Moringa oleifera* | *Zingiber officinale var. rubrum* |
| 1 | Alkaloid | Yellow | + | + |
| 2 | Flavonoid | Yellow | + | + |
| 3 | Fenol | Blue | + | + |
| 4 | Terpenoid | Red | + | + |
| 5 | Steroid | Blue | + | - |
| 6 | Tanin | Blue | + | + |

The qualitative compounds contained in moringa leaves and red ginger extracts explains the presence and absence of bioactive. The results of the tests show the presence of bioactive compounds in the form of alkaloids, flavonoids, phenols, terpenoids, steroids, and tannins in moringa leaves, with the exception of steroid compounds not found in red ginger.

**2. *In vitro* antibacteria test**

No inhibition zones were observed in any of the treatments. The negative control, which used distilled water, also showed no inhibition against the growth of the bacteria, indicating that it had no effect in the antibacterial test. The positive control was conducted to compare the diameter of the inhibition zones formed by the combinations of moringa leaves extract and red ginger (A, B, C) with 100% amoxicillin (K), a synthetic antibacterial agent, as displayed in Fig. (3).

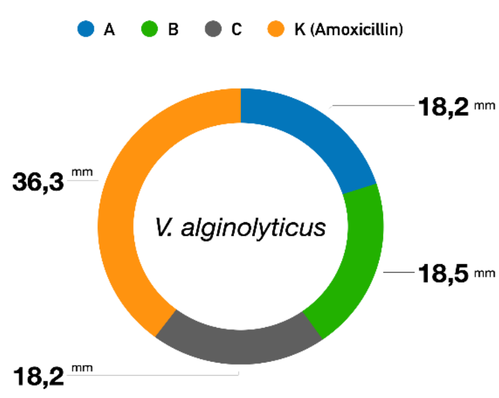


**Fig. 3.** Zone of inhibition of *V. alginolyticus* bacteria

The result shows a positive control treatment using amoxicillin produced the largest inhibition zone area measuring 36.3mm, followed by treatments B measuring 18.5mm, while treatment A and C both measured 18.2mm. This shows that the combination of moringa leaves extract and red ginger is able to provide antibacterial effects in inhibiting the growth of *V. alginolyticus* bacteria although they are not different significantly.

### 3. Response of fish to bacterial infection

The condition of fish after bacterial infection for treatments A, B, C and control indicated a change in behavioral as well as physiological traits of fish at various levels and periods. Table (2) shows the condition of fish when infected with bacteria; the clinical symptoms shows the condition of the body that secretes mucus excessively, ulcers on the skin and reddish wounds in the mouth. Fish behavior disorders happened as loss of appetite, slow movement and balance disturbed when swimming. This is a physiological response that displays the effect of bacterial attack from day 1 to day 3 for all treatments to all fish.

**Table 2.** Clinical signs of the white snapper after bacterial infection

| Day | Treatments | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | K | | | A | | | B | | | C | | |
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| **Pasca infection with *Vibrio alginolitycus*** | | | | | | | | | | | | |
| 1 | -- | ** | ** | ** | -- | ** | ** | ** | ** | ** | ** | ** |
| 2 | ** | ** | ** | ** | -- | ** | ** | -- | -- | -- | ** | ** |
| 3 | ** | ** | -- | -- | ** | -- | ** | ** | ** | ** | -- | ** |

Description:
**: Fish behavior (loss of appetite, loss of balance when swimming and moving slowly)
-- : Body condition (excessive mucus secretion, ulceration of the skin, reddish sores on the mouth)

### 4. Response of fish infected by bacteria to extract bioactive

The opposite results were observed after the immersion process with bioactive extracts: Treatment B and C achieved 100% healing success; however, for treatment K (control, with no extract application) and A, few fishes experienced death (Fig. 4).

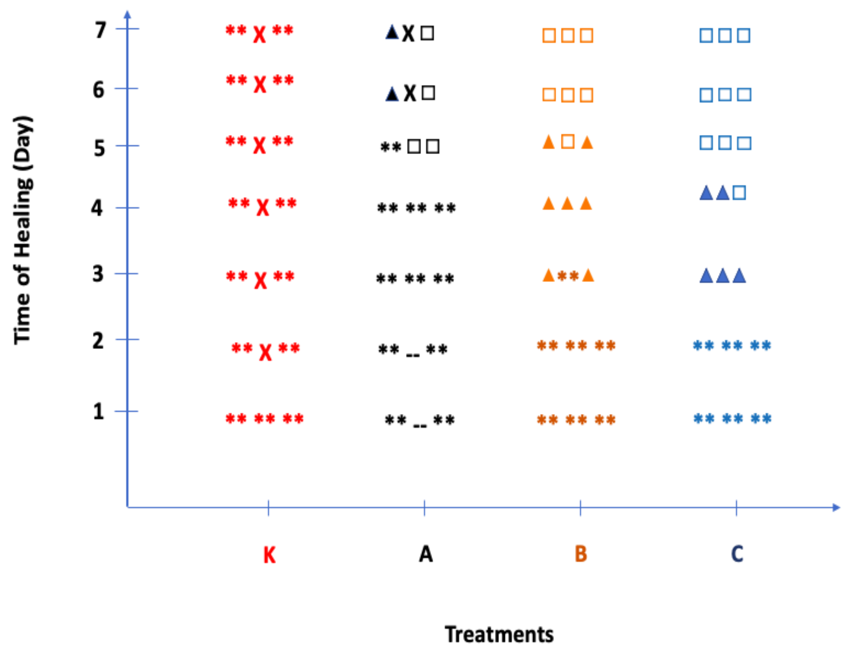


**Fig. 4.** Clinical signs of the white snapper after immersion with combination of extracts

Description:

**: Fish behavior (loss of appetite, loss of balance when swimming and moving slowly)

-- : Fish body condition (excessive mucus secretion, skin ulceration, reddish sores on the mouth)

X : fish dead

▲: fish behavior starts to be normal

□ : body condition improves

Post-treatment observations indicated deaths in treatments A (75:25% moringa and red ginger) and K (the control treatment), while treatments B and C showed improvements in body condition and a return to normal behavior starting on day 3. This suggests that the combinations of 50:50% and 25:75% moringa and red ginger extracts effectively promote recovery after infection.

**5. Survival rate of the white snapper (*Lates calcarifer*)**

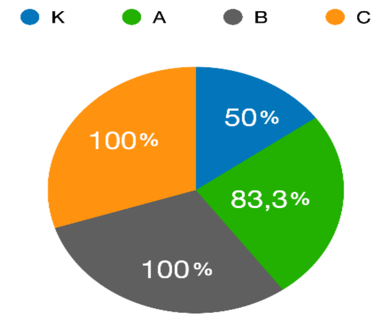


**Fig. 5.** Survival rate of the white snapper *Lates calcarifer*

Fig. (5) shows the survival rate of the white snapper in each treatment, which, if entered into the formula was 50% for treatment K, 83.3% treatment A (poor category), while treatment B and C were 100% (excellent category).

**6. Percentage of lymphocytes before and after administration of combination of extracts**

The observations revealed a decrease in the percentage of lymphocytes; however, the application of the combination of moringa leaves and red ginger extract after a Mann-Whitney U test (*P*>0,05) had no significant impact. In treatment K (control), which did not receive the extract combination, the percentage of lymphocytes ranged from 80% to 90%, with an average of 88.7%. In treatments A, B, and C, the percentages of lymphocytes were 75%–78%, 75%–77%, and 73%–76%, with averages of 76.7%, 75.7%, and 74.3%, respectively (Fig. 6).

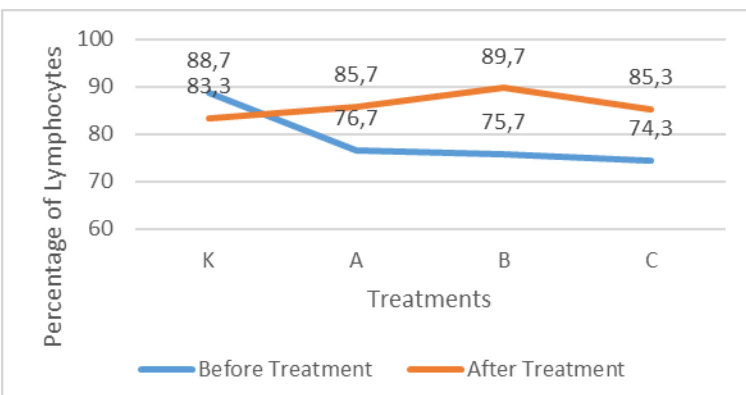


**Fig. 6.** Comparison of the white snapper lymphocytes before and after treatment

## DISCUSSION

Red ginger and moringa leaves contents of bioactive compounds have the ability to inhibit pathogens by acting as antibacterial substances **(Pattipeilohy *et al*., 2023)**. On the other hand, red ginger extract had all bioactive substances except for steroid content, moringa leaves extract contained bioactive substances such as alkaloids, flavonoids, phenols, terpenoids, and tannins. Phytochemical tests revealed the presence of several secondary metabolite compounds in red ginger extract, including alkaloids, flavonoids, phenols, tripernoids, and tannins, with no detection of the steroid test. According to the results of phytochemical tests regarding red ginger extracts conducted by **Kaban *et al*. (2016)**, red ginger contains various groups of secondary metabolic compounds, including alkaloids, flavonoids, phenolics, and triterpenoids. In the report of **Herawati and Saptarini (2020)**, it was elucidated that the red ginger rhizomes content flavonoid around 0.0068%. Due to the bioactive qualities of moringa leaves and red ginger, these two components can be utilized to decrease bacterial infections in fish, promote fish health, and reduce the harmful effects of synthetic antibiotics typically used in fish diets.

When pure water was used in the negative control test, there was no formation of the zone of inhibition. It was discovered that the inhibitory zone generated in the positive control had varying sizes. The amoxicillin control treatment had the biggest zone of inhibition, measuring the highest among the treatments, which means that the synthetic antibacterial are still stronger compared to the combination of red ginger and moringa leaves extract exhibiting a comparatively modest inhibitory zone, but this natural antibiotics are more effective and environmentally friendly **(Sembel, 2018)**. Additionally, since biopharmaceutical substances do not leave residues in the environment, using them to address food security and safety in the future is strongly advised.

After a bacterial infection, the physiological condition and health of fish undergo various changes that can affect growth, survival rates, and the quality of aquaculture products. Infected fish commonly exhibit tissue damage, red spots on the ventral and lateral areas, swollen and dark skin lesions, and the release of blood exudate, a condition known as vibriosis **(Novoa *et al.*, 1992)**. Similar findings were reported by **Seuk *et al.* (2021)** regarding the treatment of the cantang grouper (*Epinephelus fuscoguttatus-lanceolatus*) infected with *Vibrio alginolyticus* using water extracts of ketapang (*Terminalia catappa*) leaves.

Open wounds or abrasions on the host are primary means of bacterial infection, which can lead to more severe conditions such as bacteremia (blood infections). Infected fish typically experience a range of issues, from tissue necrosis to organ damage, potentially resulting in significant mortality if untreated. *Vibrio alginolyticus* utilizes its flagellum and pili to attach to host cells, where it secretes various toxins and enzymes that damage tissues, disrupt cell membranes, and evade the host's immune response. It also secretes proteases and hemolysins, which enhance its pathogenicity by breaking down proteins and causing cell lysis. This organism is well-known for being opportunistic, especially in compromised or stressed animals.

In aquaculture, *Vibrio alginolyticus* poses significant challenges due to its ability to survive in various environmental conditions, including high salinity and temperature, making it a common cause of diseases in fish and shellfish. The physiological reactions observed in fish infected in this study included redness in the mouth, increased mucus production, and skin ulcers. Similar symptoms were noted by **Cerlina *et al.* (2021)** in the dumbo catfish (*Clarias gariepinus*) treated with bay leaf solution (*Syzygium polyantha*) after infection with *Aeromonas hydrophila*.

According to **Novriadi *et al.* (2014)**, the survival rate of the white snapper is approximately 86%. However, this study indicated that a lack of treatment and lower concentrations of moringa leaf extract may lead to fish deaths. Increasing the proportion of red ginger in the extract combination showed positive results in preventing bacterial infections. **Setyowati *et al.* (2014)** explained that tannins react with cell membranes, while flavonoids alter bacterial cell membranes, causing cell coagulation and eventual rupture. Alkaloids disrupt the peptidoglycan layer in bacterial cells, hindering cell wall formation and leading to bacterial death. Essential oils can change protein structures and damage bacterial cell membranes by dissolving fats in the cell walls. Phenolic compounds form complexes with proteins through hydrogen bonds, which can also damage bacterial cell membranes. All these compounds exhibit antibacterial properties and can aid treat bacterial infections.

Mortality rates and the survival rate of the white snapper are important parameters for assessing population tolerance and viability. The survival rate (SR) can be calculated by comparing the initial number of fish at the start of the observation period with the final number at the end **(Windarto *et al.*, 2019)**.

**Hidayat *et al.* (2014)** reported that black cumin stimulated 73–77% of the white snapper's lymphocytes and was effective against *Vibrio alginolyticus*. **Blaxhall (1972)** and **Hardi (2005)** confirmed that the lymphocyte count in normal fish ranges from 71.12 to 82.88%, indicating that the lymphocyte levels in the white snapper (*Lates calcarifer*) treated with the combination of moringa and red ginger extracts remained within the normal range, despite a decrease. This combination forms antibodies that target bacteria, and lymphocytes primarily migrate to inflamed areas; thus, their numbers may decrease during recovery. Research by **Subryana *et al.* (2020)** on the use of moringa leaf extract for tilapia infected with *Aeromonas hydrophila* also showed a decrease in lymphocytes, as the antibodies act to eliminate pathogens.

In addressing diseases caused by *Vibrio alginolyticus,* various natural alternatives to antibiotics are being explored to combat antibiotic resistance and promote sustainable aquaculture practices. Herbal extracts, such as *Moringa oleifera* and *Zingiber officinale* var. *rubrum,* are known for their antimicrobial properties. *Moringa* leaf extract has proven effective against *Vibrio* species, with its compounds, such as isothiocyanates **(Lopez-Rodriguez, 2020)**, exhibiting substantial antibacterial properties. Increasing the proportion of moringa leaves in the extract combination yielded the best results for treatment. These natural approaches offer promising options for managing diseases caused by *Vibrio alginolyticus* in aquaculture while mitigating the risks associated with conventional antibiotic use.

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