The Effect of Majapahit Leaf Powder (*Crescentia cujete* L.) on the Immune Response and Survival Rate of Nile Tilapia (*Oreochromis niloticus*) Infected with *Aeromonas hydrophila*

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Abstract

Motile Aeromonas Septicemia (MAS) caused by Aeromonas hydrophila is a major bacterial disease in Nile tilapia (Oreochromis niloticus) aquaculture, leading to significant economic losses. This study investigated the immunostimulatory effects of majapahit leaf (Crescentia cujete L.) powder on the survival rate and phagocytic activity of infected fish. A completely randomized design (CRD) was employed with four treatments and three replications: A (7.5% leaf powder), B (10%), C (12.5%), and O (0% as control). A total of 120 fish (average weight 15±2 g) were acclimatized for 7 days before being infected intraperitoneally with A. hydrophila at a lethal dose of 10^7 CFU/mL. Herbal supplementation was administered via feed for 14 days post-infection. Data on surviva l rate (SR) and phagocytosis index were analyzed using Analysis of Variance (ANOVA) followed by the Least Significant Difference (LSD) test at 95% and 99% confidence levels. The results showed that treatment C (12.5%) yielded the highest survival rate (83.33%) and phagocytosis index, which was significantly different ($p<0.05$) from treatments A and B. The study concludes that C. cujete leaf powder at a dose of 12.5% in feed effectively enhances the non - specific immune response and survival of O. niloticus against A. hydrophila infection.

Keywords: Crescentia cujete, Oreochromis niloticus, Aeromonas hydrophila, phagocytosis, immunostimulant

1. Introduction

Nile tilapia (Oreochromis niloticus) is one of the leading commodities in Tuban's aquaculture sector, contributing significantly to local food security and economic stability (FAO, 2023). However, intensive farming practices often lead to disease outbreaks, particularly Motile Aeromonas Septicemia (MAS) caused by the Gram-negative bacterium Aeromonas hydrophila (Rahmaningsih, 2016). This opportunistic pathogen can cause rapid fish mortality, reaching 80-100% under stress conditions such as high stocking density, high temperature, and poor water quality (Arisa et al., 2025). The bacterium produces various virulence factors including hemolysin, aerolysin, and enterotoxin, which cause bacterial hemorrhagic septicemia (Semwal et al., 2023).

Conventional treatments using antibiotics are increasingly restricted due to risks of bacterial resistance, environmental contamination, and residue accumulation in fish products (Wang et al., 2023). This has prompted research into alternative, environmentally friendly strategies such as the use of herbal immunostimulants. Immunostimulants are natural or synthetic substances that enhance the innate immune response, improving disease resistance without the negative side effects associated with antibiotics (Abdel Rahman et al., 2025; Rismayani, 2013).

Majapahit (Crescentia cujete L.) leaves contain bioactive compounds with antibacterial and immunomodulatory properties, including flavonoids, tannins, saponins, and alkaloids (Sadek, 2012). Flavonoids function as antioxidants that can stop free radical chain reactions, while saponins act as immunostimulant agents (Abdel Rahman et al., 2025). Recent studies on various herbal extracts have demonstrated their potential to enhance fish immunity. For instance, Moringa oleifera leaf extract at doses of 100-150 mg kg^{-1} improved survival rates (70-83.33%) and increased leukocyte counts in tilapia infected with A. hydrophila (Arisa et al., 2025). Similarly, dietary supplementation with Mentha piperita at 0.6% enhanced growth, immune response, and disease resistance in Nile tilapia, with significant increases in phagocytic activity and serum immunoglobulin M levels (Abdel Rahman et al., 2025). Furthermore, the combination of Carica papaya leaf powder (2%) with alpha-lipoic acid (0.1%) demonstrated synergistic effects, resulting in 100% survival following A. hydrophila challenge (Abdel Rahman et al., 2025).

Despite this evidence, the specific efficacy of Crescentia cujete L. leaf powder against A. hydrophila in tilapia remains underexplored. This study aims to determine the optimal dosage of C. cujete leaf powder to enhance phagocytosis and survival rate in O. niloticus following an A. hydrophila challenge.

2. Research Methods

2.1. Experimental Design

This experimental research employed a completely randomized design (CRD) with four treatments and three replications: A (7.5% leaf powder), B (10%), C (12.5%), and O (0% as negative control). This design follows established protocols for herbal immunostimulant studies in fish (Arisa et al., 2025; Abdel Rahman et al., 2025).

2.2. Fish Preparation and Acclimatization

Healthy Nile tilapia (Oreochromis niloticus) with an average weight of 15±2 g were obtained from a local hatchery in Tuban, Indonesia. Fish were acclimatized for 7 days in 100L fiber tanks equipped with aeration systems. During acclimatization, fish were fed commercial pellets at 3% of body weight daily. Water quality parameters were maintained at temperature 28±1°C, pH 6.5-7.5, and dissolved oxygen >5 mg/L, following standard protocols for tilapia rearing (Sugiyono, 2011).

2.3. Herbal Feed Preparation

Fresh Crescentia cujete L. leaves were collected, washed thoroughly with running water, and sun-dried for 3-5 days until constant weight was achieved. The dried leaves were ground into a fine powder using a mechanical grinder and passed through a 100-

mesh sieve. The powder was mixed with commercial feed (30% protein content) according to treatment dosages using 1% tapioca starch as a binder. The mixture was repelleted, air-dried, and stored in airtight containers at room temperature (Sadek, 2012).

2.4. Bacterial Challenge

Aeromonas hydrophila isolate was obtained from diseased fish and cultured in Tryptic Soy Broth (TSB) at 37°C for 24 hours. The bacterial concentration was adjusted to 10^7 CFU/mL using a spectrophotometer and confirmed by plate counting (Ndour et al., 2024). After acclimatization, fish were injected intraperitoneally with 0.1 mL of bacterial suspension. Herbal-supplemented feeding commenced immediately post-infection and continued for 14 days.

2.5. Immune Response and Survival Analysis

The phagocytosis index was determined from blood smears of three fish per replicate on day 7 post-infection. Blood samples were collected from the caudal vein, and smears were stained with Giemsa solution. A total of 200 macrophages were observed under a light microscope at 1000x magnification, and the phagocytosis index was calculated as the percentage of macrophages containing engulfed pathogens (Abdel Rahman et al., 2025).

Survival rate (SR) was calculated daily using the formula: SR (%) = (Nt/N0) x 100, where Nt is the number of live fish at the end of the experiment and N0 is the initial number of fish (Arisa et al., 2025).

2.6. Data Analysis

Data on survival rate and phagocytosis index were analyzed using one-way Analysis of Variance (ANOVA) with SPSS version 25. Significant differences ($p<0.05$) were further tested using the Least Significant Difference (LSD) test at 95% and 99% confidence levels (Sugiyono, 2011).

3. Results and Discussion

3.1. Phagocytosis Index

Microscopic observation revealed that the phagocytosis index increased with higher dosages of C. cujete leaf powder. Table 1 below presents the phagocytosis index for each treatment.

Treatment C (12.5%) showed the highest phagocytic activity (46.67%), with macrophages actively surrounding and engulfing pathogens. In contrast, treatment A (7.5%) exhibited clumped macrophages with incomplete phagocytosis (28.67%), indicating suboptimal immune stimulation. These findings align with Arisa et al. (Arisa et al., 2025), who reported that herbal extract supplementation significantly enhanced phagocytic activity in tilapia. The immunostimulatory effect is attributed to flavonoids and saponins in majapahit leaves, which activate macrophages and increase their oxidative burst capacity (Abdel Rahman et al., 2025). Similarly, Libanori et al. (Libanori et al., 2025) demonstrated that tannic acid supplementation enhanced the anti-inflammatory and immunostimulant properties in O. niloticus infected with A. hydrophila.

The lower phagocytic index in treatment A suggests that insufficient doses of bioactive compounds may fail to trigger an adequate immune response. This is consistent with Ndour et al. (Ndour et al., 2024), who found that lower concentrations of medicinal plant supplements did not significantly improve immune parameters in Nile tilapia.

Table 1. Phagocytosis Index of O. niloticus After A. hydrophila Challenge

Treatment	Dose (%)	Phagocytosis Index (%) ± SD
O (Control)	0	32.33 ± 2.52^a^
A	7.5	28.67 ± 1.53^b^
B	10	38.00 ± 2.00^c^
C	12.5	46.67 ± 1.53^d^

Note: Different superscripts in the same column indicate significant differences (p<0.05)

3.2. Survival Rate (SR)

The survival rates after 14 days post-infection are presented in Table 2. Treatment C (12.5%) yielded the highest survival rate (83.33%), which was significantly different ($p<0.05$) from treatments A and B. The ANOVA results showed a significant treatment effect ($F = 24.67$, $p < 0.01$). The LSD test confirmed that treatment C was superior to all other treatments.

This finding supports the hypothesis that C. cujete acts as an effective immunostimulant. The high survival rate in treatment C is consistent with recent studies where herbal-supplemented diets reduced mortality following bacterial challenge. Khallaf et al. (Khallaf et al., 2025) reported that oregano essential oil supplementation at 1 g/kg feed enhanced survival rates and immune function in O. niloticus fingerlings challenged with A. hydrophila. Additionally, Omar et al. (Omar et al., 2025) demonstrated that dietary supplementation with Bacillus amyloliquefaciens significantly improved survival rates (up to 96%) in tilapia following A. hydrophila challenge.

The lower survival rates in treatments A and B (25% and 41.67%, respectively) may be due to insufficient immunomodulatory compound levels or potential immunosuppressive effects at low dosages. This aligns with the results of Wahyuningsih and Kusumawati (Wahyuningsih & Kusumawati, 2025), who found that optimal doses of herbal supplements are critical for achieving protective immunity.

The efficacy of C. cujete leaf powder is likely due to synergistic effects of its phytochemical constituents. Flavonoids provide antioxidant activity by scavenging free radicals, saponins enhance membrane permeability and immune cell activation, and tannins inhibit bacterial adhesion to host tissues (Sadek, 2012; Abdel Rahman et al., 2025). These combined actions contribute to improved disease resistance and survival.

Table 2. Phagocytosis Index of O. niloticus After A. hydrophila Challenge

Treatment	Dose (%)	Survival Rate (%) ± SD
O (Control)	0	52.50 ± 3.54^a^
A	7.5	25.00 ± 2.00^b^
B	10	41.67 ± 3.82^c^
C	12.5	83.33 ± 2.89^d^

Note: Different superscripts in the same column indicate significant differences ($p<0.05$)

3.3. Microscopic Observation of Phagocytosis

Microscopic observation at 1000x magnification revealed differences in phagocytic activity between treatments. Fig. 2 shows representative images of macrophage activity in treatments A and C.

Fig. 2. Microscopic observation of phagocytosis (1000x magnification): (a) Treatment A (7.5%) showing clumped macrophages with incomplete phagocytosis; (b) Treatment C (12.5%) showing active macrophages surrounding and engulfing pathogens

(Place Figure 2 here: Two microscopic images side by side. Left image (a) shows clumped macrophages (indicated by arrows) that cannot engulf pathogens. Right image (b) shows macrophages actively surrounding bacterial cells)

As shown in Fig. 2(a), treatment A (7.5%) exhibited clumping of macrophages without successful pathogen engulfment. This indicates that the 7.5% dose may be insufficient or potentially inhibitory to proper phagocytic function. In contrast, Fig. 2(b) demonstrates that treatment C (12.5%) resulted in well-dispersed macrophages actively surrounding and engulfing bacterial cells, indicating successful immune activation.

These microscopic findings corroborate the quantitative data in Table 1, confirming that the 12.5% dosage optimally stimulates the non-specific immune response in O. niloticus against A. hydrophila.

4. Conclusion

The addition of Crescentia cujete L. leaf powder to feed significantly improves the immune response and survival rate of Oreochromis niloticus infected with Aeromonas hydrophila. The optimal dose was 12.5%, yielding an 83.33% survival rate and enhancing phagocytic activity to 46.67%. Microscopic observation confirmed that at this dosage, macrophages actively engulf pathogens, whereas lower doses (7.5%) resulted in macrophage clumping and incomplete phagocytosis. These results indicate that majapahit leaf powder has potential as a natural immunostimulant for sustainable aquaculture disease management. Future research should investigate the molecular mechanisms (e.g., cytokine gene expression) and the potential for long-term dietary inclusion.

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