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The Effect of Adding Chitosan Nanoparticles Loaded with Sidr Leaf Extract (*Ziziphus spina-christi*) (CPNs SLE) in Diet on *Pangasius* sp. Infected *Aeromonas hydrophila*

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Abstract

One of the bacterial types that affects freshwater fish is *Aeromonas hydrophila*. These bacteria cause the occurrence of Motile Aeromonas Septicemia (MAS) disease. This study investigates the impact and optimal dosage of chitosan nanoparticles (CNPs) loaded with sidr leaves extract (SLE) on clinical manifestations, growth, hematological parameters, and survival rates of Pangasius sp. infected *A. hydrophila*. The experimental design employed a Completely Randomized Design with five treatments and three replications, utilizing 150 fish with an average length of 7-9 cm. Treatments included the addition of 10 ppm tetracycline (K+) in the diet and CNPs SLE with concentrations A (0 ppm), B (450 ppm), C (500 ppm), and D (550 ppm). The results demonstrate that incorporating CNPs SLE into the diet yields beneficial effects on infected *Pangasius* sp., leading to faster recovery from clinical symptoms, enhanced growth rates, and increased survival rates of up to 85%. The optimal dosage of CNPs SLE for *Pangasius* sp. infected *A. hydrophila* in the feed is determined to be 500 ppm.

Keywords: Sidr leaf, *A. hydrophila*, Chitosan Nanoparticles. *Pangasius* sp., blood profile

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1. Introduction

Motile Aeromonas Septicaemia (MAS) represents a significant threat to freshwater fish populations, resulting in substantial economic losses due to its alarmingly high mortality rates, which can reach 80-100% within a short timeframe of 1-2 weeks [1]. Treating infected fish incurs elevated production costs. Data from The Ministry of Maritime Affairs and Fisheries (2022) highlight the widespread occurrence of MAS across 18 provinces in Indonesia, ranking it as the second most prevalent among 13 distinct fish diseases [2]. Importantly, the indiscriminate use of antibiotics such as Tetracycline, Nitrofuran, and Chloramphenicol during MAS treatment can foster the emergence of drug-resistant pathogenic bacteria [3]. Nanotechnology represents a scientific domain with immense potential for generating novel and cutting-edge materials [4]. This field employs nanoscale materials, typically ranging from 1 to 100 nm in size [5]. The prowess of nanotechnology resides in its capability to protect loaded drugs or compounds from enzymatic degradation, thereby ensuring their safe delivery to target organs. Moreover, it enables controlled and sustained release, extending their duration within the body, and can harmoniously integrate with other therapeutic modalities, leading to the pursuit of an optimized healing strategy [6]. Chitosan is a prominent material in bionanotechnology due to its immunomodulatory properties. Chitosan effectively enhances the immune response of Pangasius sp. infected with *A. hydrophila*, highlighting its potential in disease management [7]. Transforming chitosan into chitosan nanoparticles (CNPs) further improves its bioavailability and drug delivery capabilities, making it an efficient carrier for therapeutic compounds. Nanochitosan not

only aids in medicinal applications but also acts as a valuable supplement carrier, contributing to overall fish health. The role of CNPs as beneficial additives in fish feed, stimulating growth, boosting immune defenses, and reducing pathogenic bacterial proliferation in the gastrointestinal tract [8]. Previous research has revealed compelling evidence showcasing the bactericidal properties of chitosan nanoparticles (CNPs) against *A. hydrophila* bacteria [9]. This notable effect is ascribed to CNPs adhering to and disrupting bacterial cell walls, ultimately leading to cell lysis and death. Furthermore, the efficacy of CNPs can be significantly enhanced through the encapsulation of active compounds. Numerous investigations have been undertaken to assess the advantages of both pristine chitosan nanoparticles (CNPs) and CNPs loaded with various substances. The integration of active compounds within CNPs provides distinct benefits, such as improved compound stability during storage and controlled release within the body [10]. Moreover, other research demonstrated that supplementing fish feed with active compounds combined with CNPs yielded superior results compared to treating these compounds or CNPs individually [11]. The management of *A. hydrophila* can be achieved by administering herbal ingredients possessing antibacterial properties, such as sidr leaves. Sidr leaf extract (SLE) exhibits antibacterial effects due to its content of flavonoids, alkaloids, saponins, tannins, and steroids [12]. The incorporation of bioactive compounds via oral feed is considered an optimal approach in fish farming. This method is favored for its simplicity, time efficiency, and minimal stress imposed on the fish. Furthermore, the digestive tract serves as an ideal site for administering these compounds, as it exploits the lymphoid tissue within the mucosal lining, effectively stimulating the fish's immune response [13].

This study is aimed at analyzing the impact of chitosan nanoparticles (CNPs) loaded with sidr leaves extract (SLE) on the health status and survival rate of Pangasius sp. infected with *A. hydrophila*, with the goal of determining the optimal dosage for combating bacterial infection.

2. Materials and methods

2.1. Sample Preparation

The dried sidr leaves utilized in this study were sourced from Sragen Regency, Central Java. A total of 500 grams of dried sidr leaves were mixed and stirred with 5 liters of 96% ethanol for 5 minutes and then left to macerate for 4 days in a dark environment to facilitate the maceration process. Subsequently, the mixture was filtered using filter paper and evaporated using a rotary evaporator [14]. The production of CNPs SLE followed the ion gelation method using tripolyphosphate (TPP) [14]. To prepare 100 ml of CNPs SLE, 160 mg of chitosan was dissolved in 80 ml of 1% acetic acid using an overhead stirrer at 1000 rpm and 60°C for 2 hours. Additionally, 40 mg of TPP and 80 mg of sidr leaves extract were dissolved in 20 ml of distilled water with an overhead stirrer at 1000 rpm at room temperature for 1 hour. The TPP was then slowly added to the chitosan solution dropwise using a burette while continuously mixing with a stirrer for 30 minutes. The pH of the solution was adjusted to 6 by adding NaOH to neutralize it. The CNPs SLE solution was stored in a refrigerator for a maximum of 30 days to maintain its quality. The fish diets containing tetracycline and CNPs were prepared using commercial feed, which was initially crushed into a powder using a hammer mill. The fish diets, comprising 10 ppm tetracycline antibiotics and a specific concentration of CNPs SLE, along with 100 grams of feed, were mixed in 60 ml of distilled water using a mixer. The mixture was then processed into feed diets using a 2 mm diameter meat grinder and dried in an oven at 50°C for 1 hour [15]. For optimal results, the fish diets containing CNPs were stored for 3 months at room temperature, 6 months at 40°C, and 12 months at 25°C [16]. The bacterial strain used in this study was *A. hydrophila*, which had undergone three passages to enhance its virulence.

2.2. Research Design

150 individuals of *Pangasius* sp. were utilized in this study. The juvenile specimens, measuring approximately 8-10 cm in length, were sourced from Mranggen, Demak Regency, Central Java, and were deemed healthy. The fish underwent a 7-day acclimatization period under controlled conditions to ensure their health status. Health was determined based on the absence of clinical signs in terms of physical appearance and behavior. Subsequently, the fish were challenged with *A. hydrophila* at a dosage of 0.1 ml per fish, with the bacterial density determined from the LD50 test at 10^8 CFU/ml intramuscularly.

A Completely Randomized Design comprising 5 treatments and 3 replications was applied in this research:

K = Diet added 10 ppm tetracycline
A = Diet without addition of CNPs SLE
B = Diet added 450 ppm CNPs SLE
C = Diet added 500 ppm CNPs SLE
D = Diet added 550 ppm CNPs SLE

The fish were fed with a diet containing CNPs SLE and tetracycline twice daily, at 08:00 in the morning and 16:00 in the afternoon, using the satiation method. This feeding regimen was maintained for 28 days following the onset of clinical signs associated with *A. hydrophila* infection in the fish. Water quality parameters, including water temperature, pH, and dissolved oxygen (DO), were monitored daily. Blood parameters were assessed at specific time points: before treatment and infection (day 0), the first day after clinical signs were observed, and on days 7, 14, 21, and 28.

2.3. Examination of Hemotologycal Profile

The blood collection method, involves blindfolding the fish and covering it with a wet cloth to minimize stress. Blood is collected near the caudal fin using a 1 ml syringe previously washed with EDTA. Approximately 0.1 ml of blood is transferred to a microtube and thoroughly shaken to prevent agglutination. Following blood collection, the fish is exposed to strong aeration and then returned to the aquarium [17]. Hemoglobin (Hb) levels are determined following the protocol outlined by Simmons (1980), involving the dissolution of blood in 10N HCl, subsequent dilution with aquadest, and color matching with an indicator. The total red blood cell (RBC) and white blood cell (WBC) counts per mm3 are determined using a Neubauer hemocytometer and calculated using the formula on bellow. The calculation of the total RBC count is conducted as follows [18]:

$$RBC\ (cells/mm^3) = N \times 50$$

The total WBC count was calculated as follows:

$$WBC\ (cells/mm^3) = N \times 10^4$$

Where: N is initial of total number of calculated cells.

The percentages of differential leukocytes were determined using blood smears from the fish. To prepare the smear, a small drop of blood was placed on the glass slide and spread evenly, followed by staining with Diff Quick dye. Subsequently, the differential leukocytes were counted using a microscope at 400x magnification.

2.4. Growth Parameters

The fish weighted every 2 weeks and calculated the growth (WG) using formula as follows:

$$WG = W_{f(g)} - W_{i(g)}$$

The Relative Growth Rate (RGR) calculated as follows:

$$RGR = \frac{W_{f(g)} - W_{i(g)}}{W_{i(g)}} \times t$$

Where: W_f = final body weight; W_i = body weigh at the beginning; t = rearing period days

Survival Rate (SR)

The survival rate of the fish along 28 days was calculated using formula as follows:

$$SR(\%) = \frac{N_t}{N_0} \times 100$$

Where: N_t is number of fish that survived at the end of the experiment; N_0 is number of fish at the beginning of the experiment

2.5. Statistical Analysis

The data were summarized as the mean and standard deviation. Statistical analysis was conducted with a significance level of $p<0.05$ using an analysis of variance test performed in SPSS for Windows. Dunnet's test was employed to compare significant differences with the control treatment.

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3. Results and Discussions

3.1. Result

3.1.1. Clinical Signs

Clinical symptoms manifest in Pangasius sp. infected with *A. hydrophila* approximately 36 hours post-infection, including reduced appetite, lethargic swimming, red spots, exophthalmia, ulcer formation, and dropsy in the abdominal region (refer to Figure 1). Some infected fish may succumb to mortality due to these symptoms. Subsequently, the fish receive treatment feed as per the prescribed regimen. The recovery process for *Pangasius* sp. begins around the 7th day, characterized by a reduction in red spots and gradual wound healing. Both treatment K+ and treatment C show improved appetite. By the 12th day, fish treated with K+ exhibit no remaining clinical symptoms. However, fish under treatment A still display redness, mucus secretion, and ragged fins. Notably, treatment B shows enhanced fin condition. Treatments C and D demonstrate a visible decrease in fish redness, along with healed wounds. The progress in wound healing among Pangasius sp. fed with CNPs SLE in treatment C is illustrated in Figure 2.

3.1.2. Blood Parameters Observation

The results of blood profile observations, including total erythrocytes, total leukocytes, and hemoglobin, on days 0, 1, 7, 14, 21, and 28 are depicted in Figure 3. Figure 3 illustrates an increase in the mean total leukocyte count on day 1 following *A. hydrophila* infection, followed by a decrease on day 14 across all treatments. Total erythrocytes and hemoglobin levels fluctuated from day 0 to day 21, with an increase observed in all treatments on day 28.

3.1.3. Leukocytes Differentials

Figure 4 presents the percentages of lymphocytes, monocytes, and neutrophils in *Pangasius* sp. over the 28-day treatment period. On day 0, lymphocyte percentages ranged between 84-90%, monocytes between 6-8%, and neutrophils between 3.67-7.67%. Post- *A. hydrophila* infection (Day 1), lymphocyte percentages decreased while monocytes and neutrophils increased across all treatments. Subsequently, lymphocyte percentages increased again, while neutrophils and monocytes decreased starting from day 14, indicating fish recovery.

3.1.4. Growth Parameters

The weight gain (WG) and relative growth rate (RGR) of the fish after the 28-day treatment are presented in Table 1. Table 1 indicates that the highest WG and RGR were observed in treatment C (500 ppm CNPs SLE), while the lowest values were recorded in treatments D (550 ppm CNPs SLE) and A (0 ppm CNPs SLE). According to the ANOVA test results, significant differences were observed in WG and RGR among the different doses of CNPs SLE and the positive control. Subsequently, Dunnet's test was performed to compare with the control treatment, revealing that only treatment C significantly differed from the positive control treatment.

3.1.5. Survival Rate (SR)

Figure 5 illustrates the survival rate of the treated fish over 28 days. The outcomes indicate significant differences ($p < 0.05$) between treatments A and C compared to treatment K+. Treatment C (500 ppm CNPs SLE) achieved the highest survival rate among all treatments, while the lowest was observed in treatment A (feed without CNPs SLE).

3.1.6. Water Quality

Table 2 displays the water quality parameters monitored during the treatment. The temperature, pH, and dissolved oxygen (DO) levels in this study remained within optimal ranges for the life of *Pangasius* sp. and *A. hydrophila*.

3.2. Discussions

3.2.1. Clinical Signs

Clinical signs of *A. hydrophila* infection typically manifest 36 hours post-infection in fish. These findings align with previous research who noted that Pangasius sp. infected with *A. hydrophila* start displaying clinical symptoms 24 hours post-infection [19]. Decreased appetite is characterized by a reduced feeding response and lower feed consumption compared to healthy fish. This decline in appetite is attributed to stress and disruption of the fish's digestive system following *A. hydrophila* infection [20]. Past research highlighted that *A. hydrophila* releases enterotoxins targeting the digestive tract, causing damage [3]. This is corroborated by the presence of redness around the anus and the occurrence of dropsy as shown in Figure 1. Dropsy begins to manifest on the 2nd day across all treatments, evidenced by the enlargement of the fish's abdominal cavity due to stagnant body fluid accumulation, attributed to Aerolysin Cytotoxic Enterotoxin [21]. Reddish spots appear on the body and head surface of *Pangasius* sp. approximately 36 hours post-

infection with *A. hydrophila* in all treatments. This is caused by blood vessel rupture, resulting in blood leakage and surface redness [3]. Ulceration is characterized by inflammatory response enlargement into a wound and hemorrhaging at the *A. hydrophila* injection site. Bacterial infection in fish triggers an immune response, leading to polymorphonuclear leukocyte activation as phagocytic cells [22]. This, in turn, prompts bacterial hemolysin production, which is toxic and contributes to ulceration and hemorrhaging in the fish's body. The formation of ulcers is attributed to the elevated bacterial density concentrated at specific sites, as observed in this study at the injection site of *A. hydrophila* [23]. Consequently, the concentration and potency of toxins released by the bacteria at that site are heightened. Exophthalmia begins to manifest on the 2nd day, characterized by the enlargement and protrusion of the fish's eyes. As indicated by the previous research, exophthalmia results from gas production by *A. hydrophila*, leading to gas accumulation within the fish's eyes [3]. The recovery process of fish following infection with *A. hydrophila* commenced on day 7 post-treatment initiation, characterized by the gradual closure of ulcers and reduction in redness. By the end of the 14th day, fish treated with K+ exhibited complete wound closure. In contrast, fish in treatment A (0 ppm) with existing wounds succumbed to mortality, while others continued to exhibit clinical symptoms such as redness, exophthalmia, and frayed fins. Fish treated with CNPs SLE displayed ongoing clinical symptoms but demonstrated a recovery trend in terms of fin condition, redness reduction, and wound inflammation. These findings highlight tetracycline treatment as the most effective in alleviating clinical symptoms in *A. hydrophila-i*nfected fish, followed by CNPs SLE treatment. The restorative effect of CNPs SLE is attributed to its antibacterial properties. SLE confers health benefits due to its antibacterial, antioxidant, and anti-inflammatory properties. The observed reduction in inflammation is attributed to the presence of anti-inflammatory compounds in CNPs of SLE [24]. The anti-inflammatory compounds in SLE, including Neophytadiene, a terpenoid derivative [12]. Previous research support this by affirming the anti-inflammatory activity of Neophytadiene compounds and their role in wound healing [25].

3.2.2. Blood Profile

Hematological parameters, including red blood cell (RBC) count, white blood cell (WBC) count, hemoglobin (Hb) level, and differential leukocyte count, serve as key indicators for assessing fish health status [26]. At the onset of the experiment (day 0), the average WBC count of fish ranged from 1.29 to 1.62 x 10^4 cells/mm^{3,} a value considered within the normal range [3]. Subsequently, the WBC count increased to 1.43 to 2.24 x 10^4 cells/mm^3 on day 1 following *A. hydrophila* infection, observed across all treatments. This elevation in total leukocyte count correlates with the presence of *A. hydrophila* infection, prompting the fish's immune response to activate leukocyte subtypes such as neutrophils, lymphocytes, and monocytes [1]. A reduction in total leukocyte count is observed on the 14th day of treatment across all experimental groups. This decline in leukocyte levels signifies the cessation of the fish's immune response against bacterial infection. The constituents present in sidr leaves (*Z. spina-christi*), such as cyclopeptide alkaloids, tannins, phytosterols, flavonoids, triterpenoid sapogenins, and saponins, contribute to improved blood profile parameters [27]. Sidr leaves (*Z. spina-christi*) are traditionally utilized for their antimicrobial, immunostimulant, hepatoprotective, and medicinal properties. Combining the benefits of sidr leaves with CNPs can enhance the bioavailability of active compounds in sidr leaves, facilitating their rapid absorption and utilization by the fish's physiology.

3.2.3. Leukocytes Differentials

The initial lymphocyte count in the fish was notably elevated compared to the normal range of lymphocytes in fish [28]. Monocyte and neutrophil levels at the onset were within the normal range, approximately 6.00-8.33% and 3.67-7.67%, respectively [29][30]. A decrease in lymphocyte percentage was observed on the first day across all treatment groups (post *A. hydrophila* infection). This reduction in lymphocytes suggests an elevation in antibodies targeting the bacteria, resulting in a decline in the population of immune response mediators (lymphocytes) [31]. From day 14 to 28, lymphocyte levels in all treatments increased again, accompanied by a decrease in the percentage of other leukocyte differentials. This indicates that the fish's immune system has effectively responded to the bacterial challenge. The percentage of neutrophils and monocytes in *Pangasius* sp. increased on the first day following infection with *A. hydrophila*. This elevation in monocytes and neutrophils levels signifies the presence of a bacterial infection, triggering an enhanced production of monocytes aimed at bacterial destruction [28][32]. Monocytes serve as precursors to macrophages and play a pivotal role in the phagocytic mechanism against bacterial infections [33]. According to the previous research, neutrophils act as the frontline of the non-specific (innate) immune system in combating infectious diseases [34]. Neutrophils release antibacterial molecules and neutrophil extracellular traps (NETs) as barriers to infection. By day 14, all treatments exhibited a decrease in monocyte numbers. This decline in monocytes and neutrophils levels indicates either a recovery process in the fish or a diminishing infection, resulting in reduced demand for phagocyte cell production [28][35].

3.2.4. Growth Parameters

According to Table 1, it was observed that the highest Weight gain (WG) and Relative Growth Rate (RGR) were achieved in Treatment C, followed by Treatment B, A, and the lowest in Treatment D. Weight gain is associated with the antibacterial content present in Treatment K+ and the addition of CNPs SLE, enabling the energy derived from the feed to be utilized not only for combating bacterial infections but also for fish growth. Weight gain can be attributed to the antibacterial content, which enhances nutrient absorption and can stimulate growth. By the end of the experiment, improved fish health was also evident, as evidenced by the absence of clinical symptoms [36]. The results of the study also demonstrate that the application of CNPs leads to superior weight gain (WG) compared to feeding without any additives. These findings are consistent with the previous research, indicating that adding CNPs to fish feed promotes better growth than feed without CNPs [11]. This is because CNPs aid in improving nutrient absorption, combating bacteria, and increasing the activity of digestive enzymes that contribute to fish growth.

Table 1: The Influence of CNPs SLE in Feed on Fish Growth

Treatment	WG	RGR
K+	2.06 ± 0.86	1.18 ± 0.38
A	1.78 ± 0.38	1.09 ± 0.29
B	2.33 ± 0.33	1,48 ± 0.30
C	3.28 ± 0.25	2.14 ± 0.39
D	1.39 ± 0.25	0.93 ± 0.30

Table 2: Water Quality

Treatment	Temperature (ºC)	pH	Dissolved Oxygen (mg/L)
K+	25.1 – 27.5	6.64 - 8.52	4.2 - 7.5
A	25.0 - 27.7	6.68 - 8.59	4.9 - 7.8
B	25.0 - 27.6	6.94 - 8.57	4.8 - 7.6
C	25.1 - 27.6	7.04 - 8.49	4.8 - 7.7
D	25.1 - 27.5	7.02 - 8.35	4.6 - 7.8

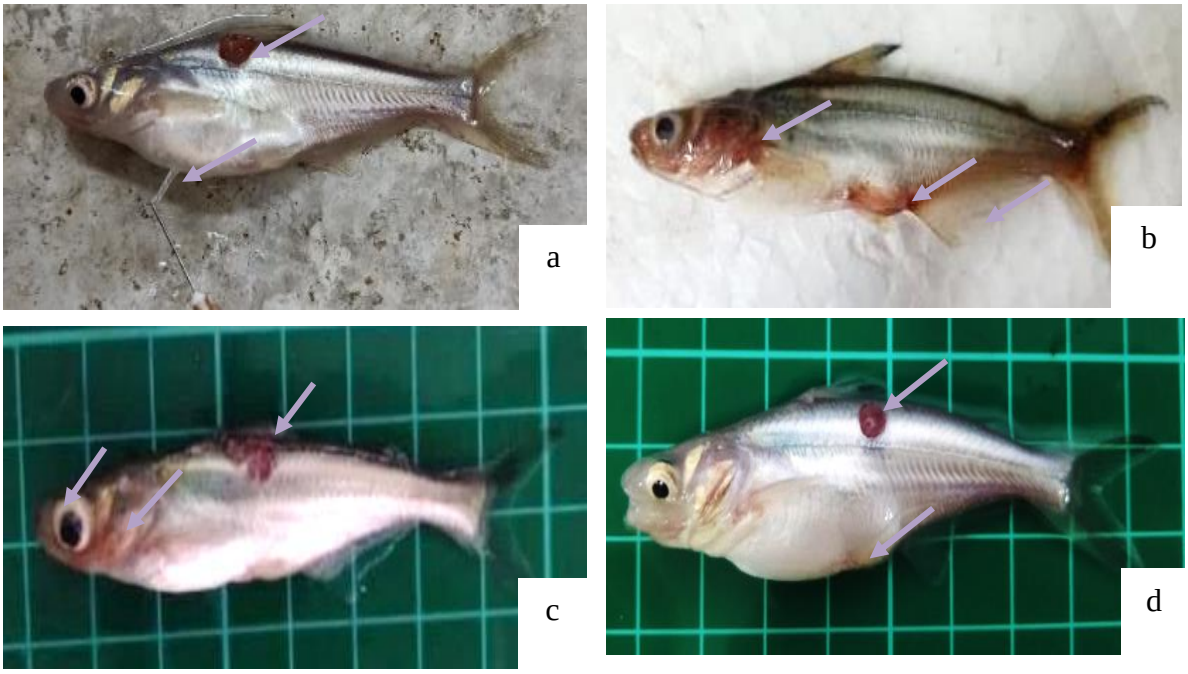
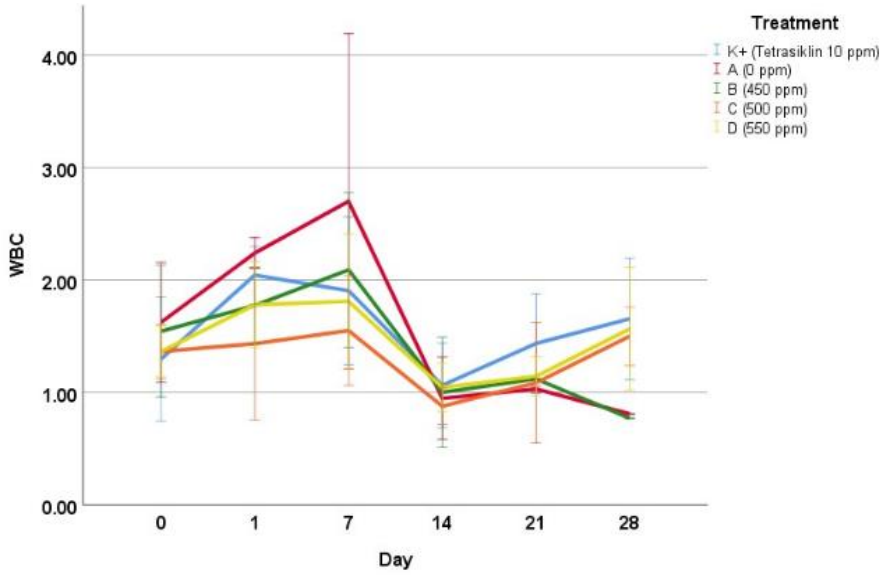


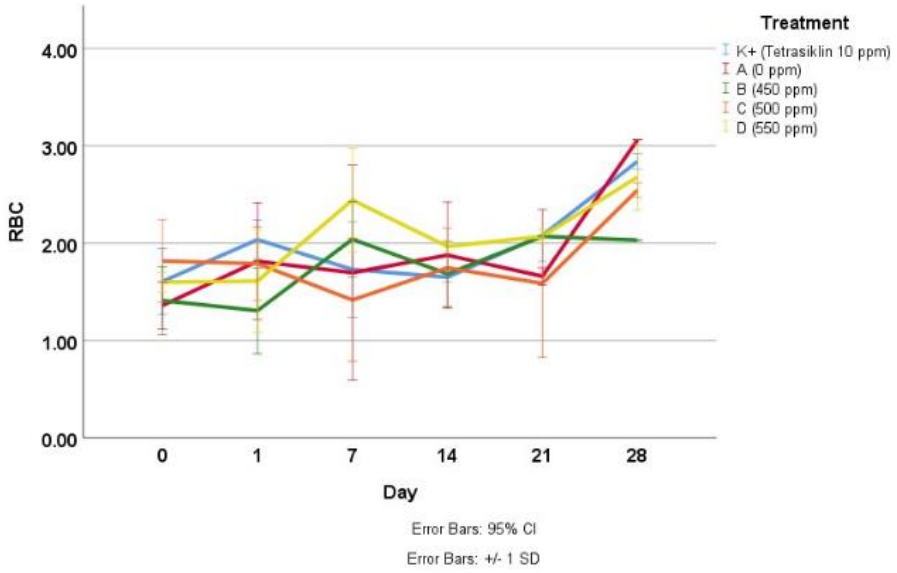
Figure 1. Clinical Signs of *Pangasius* sp. after Infected by *A. hydrophila*
(a) 36 hours post-infection, excessive mucus production, and wounds; (b) On the second-day post-infection, exophthalmia, reddish spots, wounds, and frayed fins are observed; (c) Exophthalmia, wounds, and redness; (d) Wounds and dropsy.

Figure 2. The Wound Healing Process in *Pangasius* sp. Infected with *A. hydrophila* after Treatment
(a) 36 hours post infection, opened and reddened wound; (b) D-7, inflammation in the wound reduces; (c) D-14, the wound starts to close

(A)



(B)



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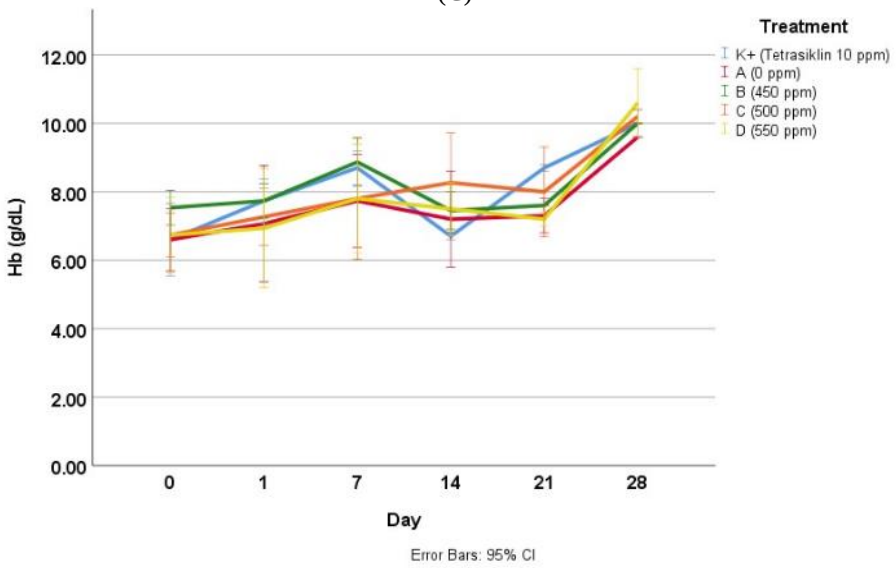
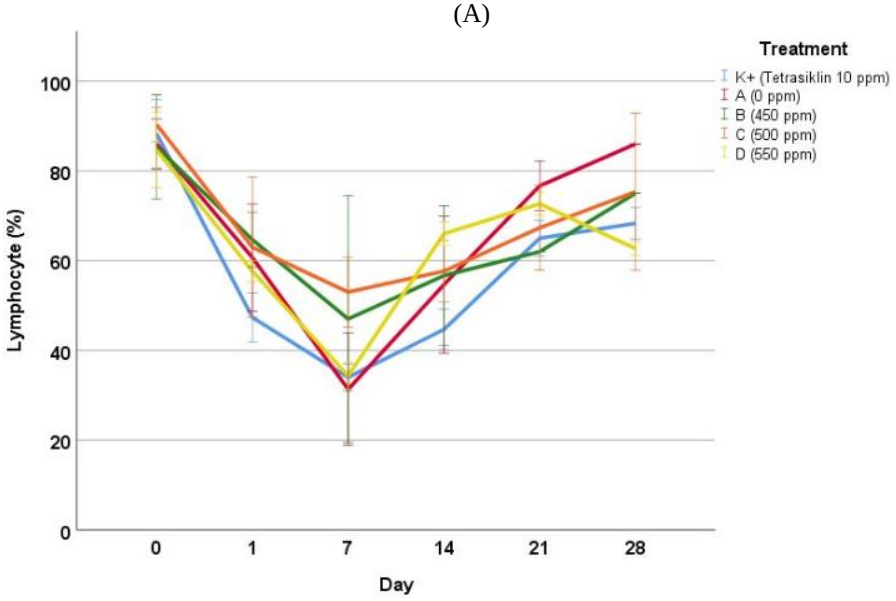


Figure 3. Blood Profile Chart a) WBC, b) RBC, c) Hb

(A)



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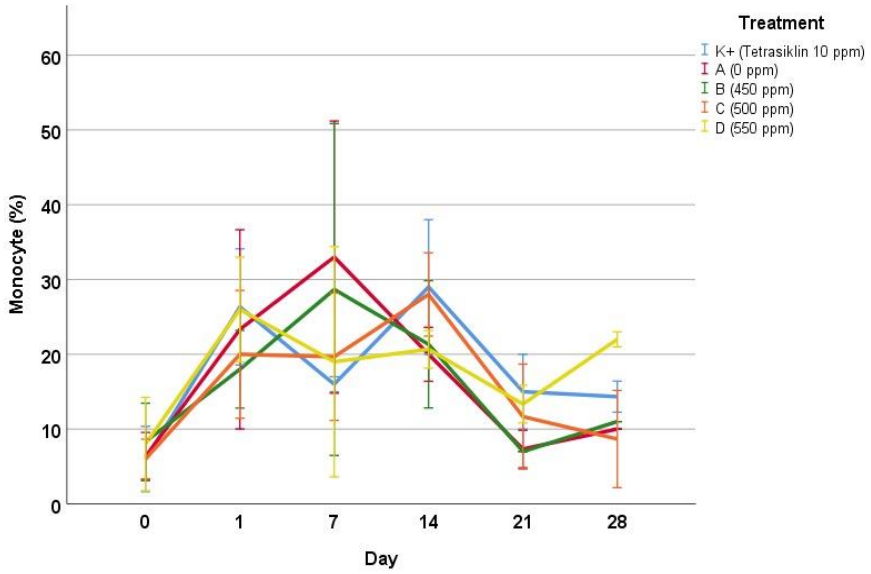

(C)

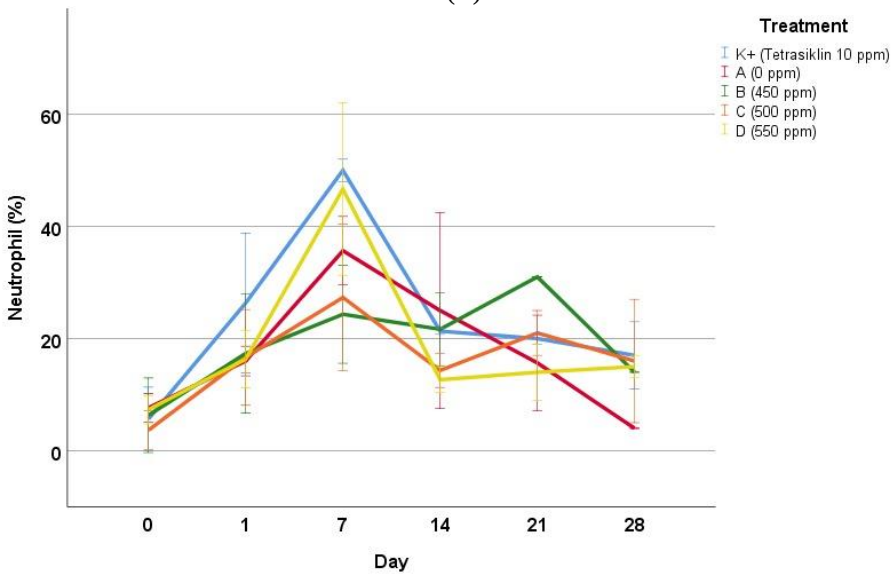

Figure 4. Differential Leukocytes Count a) Lymphocytes, b) Monocytes, c) Neutrophils

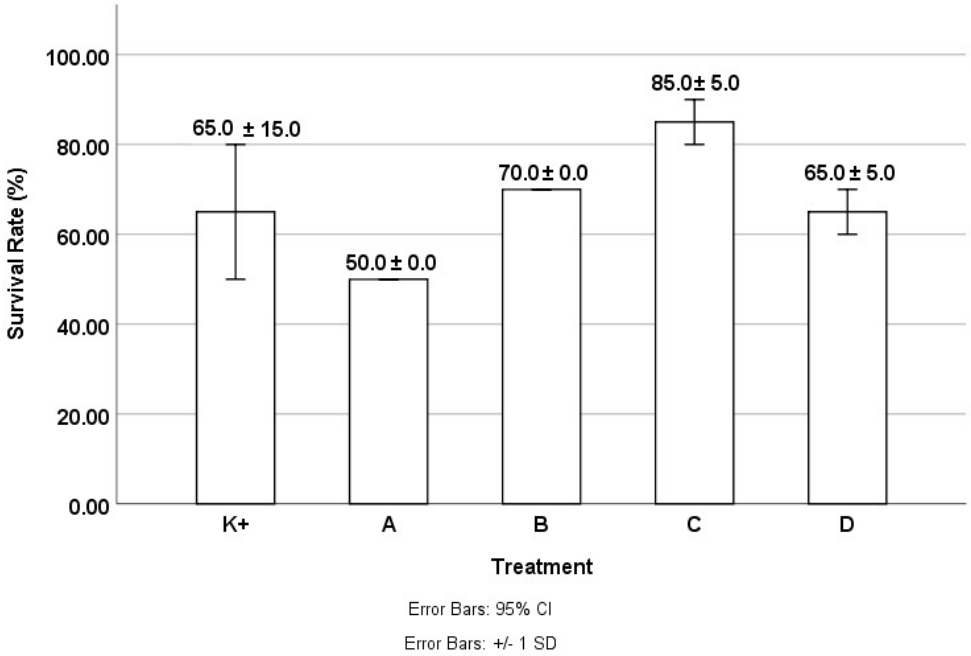

Figure 5. Graph of *Pangasius* sp. Survival Rate (%)

Histological observations conducted the previous research on snapper fish reveal that adding chitosan to the feed stimulates microvilli growth in the digestive tract [37]. This increases the nutrient absorption area, influencing overall organ development. However, research findings suggest that increasing CNPs SLE concentration does not result in greater WG and RGR. This may be due to hindered digestion caused by excessive microvilli growth in the digestive tract [38].

3.2.5. Survival Rate (SR)

The results demonstrated a significant effect ($p<0.05$) of CNPs SLE with varying doses on the survival of *Pangasius* sp. Additionally, treatment A and C exhibited significant differences compared to treatment K+. This disparity arises from treatment A lacking CNPs SLE supplementation, while treatment C, enriched with CNPs SLE, proved to be the most effective. All CNPs SLE-inclusive treatments showed improved survival rates compared to treatment A, attributed to the presence of phytochemicals like flavonoids, saponins, and tannins in CNPs SLE. Antibacterial compounds such as phenols, flavonoids, and alkaloids [12] act by destroying the cell membrane's integrity and triggering intracellular leakage [39]. This is done by accumulating hydrophobic groups on the phospholipids, causing cell death. Phenolic components can inhibit cell DNA and RNA synthesis [39, 40]. These due to antioxidants and antibacterial properties of extracts from sidr leaves (*Ziziphus Spina*-christi) [40].

A similar mechanism is also employed by CNPs in combating bacteria. The antibacterial mechanism of CNPs involves attaching to the outer membrane of bacteria, subsequently degrading and damaging the bacterial cell membrane, leading to cell shrinkage [41]. The bacterial cell then ruptures, ultimately resulting in cell death. Additionally, CNPs also possess the ability to stimulate the immune system and act as antioxidants in fish [42]. The antioxidant content present in CNPs SLE can counteract free radicals and protect cellular components from damage, as well as aid in accelerating the healing process [43]. The natural antioxidant system within the fish's body is supported by additional antioxidants derived from chitosan and plants [44]. Loading the extract of sidr leaves (SLE) into CNPs results in a synergistic effect due to their shared antibacterial properties. Chitosan also stimulates the fish's immune system as it contains amino moieties that leukocytes recognize to trigger an immune response [45]. The benefits provided by chitosan and compounds from sidr leaf extract become more effective due to the small size of CNPs. The small size of CNPs facilitates their absorption by fish hepatocyte cells, thereby enhancing antioxidant capacity and activity [46].

4. Conclusions

The addition of chitosan nanoparticles from sidr leaves extract (CPNs SLE) in diet can enhance the survival rate of *Pangasius* sp. infected with *A. hydrophila*. The best concentration of CNPs SLE in the diet is 500 ppm, with a survival rate reaching 85%. Therefore, this study found that carbon nanoparticles chitosan loaded with sidr leaf extract could (CPNs) be an alternative technology for combating *A. hydrophila* in fish.

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