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Impacts of a Diet Supplemented with *Terminalia catappa* Leaf Powder on the Histopathological Changes in Ornamental Fish (*Poecilia sphenops*) Infected with *Aeromonas hydrophila*

Harish A.[1], Raja Jeya Sekar R.[1]*, Vijila S.M.[2] and Maharajan A.[3]

[1]P.G. & Research Department of Zoology, S.T. Hindu College (Affiliated to Manonmaniam Sundaranar University, Tirunelveli) Nagercoil 629002, Kanyakumari , Tamil Nadu, India
[2]Department of Zoology, Pioneer Kumaraswamy College (Affiliated to Manonmaniam Sundaranar University, Tirunelveli) Nagercoil 629003, Kanyakumari , Tamil Nadu, India
[3]P.G & Research Department of Zoology, Khadir Mohideen College (Affiliated to Bharathidasan University, Tiruchirappalli), Adiramapatinam 614701, Tamil Nadu, India

**Corresponding Author*

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Abstract: The present study aims to evaluate the impact of *Termanalia catappa* leaf powder on the growth and resistance of Molly fish (*Pocelia sphenops*) to freshwater bacteria *Aeromonas hydrophila*. Fishes with an average weight of 2.85 ± 0.06 g were fed diets containing six different concentrations of *T. catappa* leaf powder over a 30-day period. Growth parameters of the fish were assessed during this period and after this feeding period, followed by exposure to *A. hydrophila*. Survival rates of infected fishes were recorded after a 10-day challenge test. The results indicated that the group fed with the highest concentration of *T. catappa* [AD5] exhibited significantly higher specific growth rate (SGR) and weight gain (WG) compared to other diet groups. Moreover, 80 % of the fishes in the AD5 group survived the bacterial infection challenge. Histopathological analysis of fish gills revealed severe damage in groups fed with lower concentrations of *T. catappa* leaf supplemented diet while those fed with high concentrations of diet [AD5] showed a recovered gill structure with a few observable damages. These findings suggest that dietary supplementation with *T. catappa* leaves, particularly at higher doses can enhance the growth of *P. sphenops* fish and provide protection against *A. hydrophila* infection, potentially benefiting ornamental fish farming.

Keywords: *Termanalia catappa, Pocelia sphenops*, *Aeromonas hydrophila*, Growth parameters, Histopathology

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Introduction

The cultivation of ornamental fish is emerging as a significant aspect of India's fisheries industry, alongside the production of food fishes, contributing to employment opportunities and export revenues. India possesses considerable potential in the realm of ornamental fish farming, reflecting the country's capacity for this sector's growth and development (Ghosh *et al*., 2003). This industry is rapidly expanding because of its attractiveness and the lucrative export opportunities it offers to various countries (Ahilan *et al*., 2013). The ornamental fish cultivation sector incurs substantial economic losses due to the high mortality rates resulting from outbreaks of various bacterial diseases (Austin *et al*., 2007). The gram-negative bacterium *Aeromonas hydrophila* is responsible for various disorders, including red fin disease, motile Aeromonas septicemia, and hemorrhagic septicemia. (Bisht *et al*., 2016). Various synthetic drugs are employed to treat these bacterial diseases, but they are frequently expensive and lack effectiveness. Additionally, the use of synthetic medications carries environmental risks, such as biomagnification and the development of resistant bacterial strains (Citarasu *et al*., 2002). The dispersion of antibiotics into aquatic environments can have detrimental effects on other organisms (Kim *et al*., 2009).

Plant-derived extracts are utilized to treat a variety of fish diseases, aiding in the support of natural behaviour and immunity while also helping to prevent the spread of infections (Sheeba *et al*., 2023). The herbal plants are the best source of antibiotic alternatives for diseases prevalent in aquaculture (Van Hai and Ngo, 2015). The derivatives from various herbal plants contribute to enhancing the immune system of fish (Giri *et al*., 2015).

Dried leaves of *Termanalia catappa* serve as a substitute for antibiotics in managing fish pathogens (Chitmanat *et al*., 2005). Additionally, the leaves are reported to possess antioxidant and anti-clastogenic properties (Masuda *et al*., 1999). *Terminalia catappa* has been reported to contain numerous compounds of therapeutic value, including hydrolyzable tannins such as punicalagin, punicalin, terflavins, tergallagin, tercatain, chebulagic acid, geraniin, and granatin. Additionally, it contains flavonoids and triterpenoids (Liu *et al*., 1996).

Histopathological analysis of fish organs serves as a valuable tool for promptly identifying illnesses. This analysis is particularly advantageous for detecting infections caused by pathogens in fish organs during the early stages of disease development (Takashima and Hibiya, 1995; Genten *et al.,* 2009). Pathogens, toxins, unfavourable nutritional and environmental conditions, along with various stressors, collectively influence histopathological alterations in fish. Moreover, histopathological biomarkers serve as reliable indicators of environmental stress, as they consider biotic factors and water quality, providing a comprehensive assessment of the fish's condition (Segner and Braunbeck, 1988; Handy *et al*., 2002; Stentiford *et al*., 2003; Zimmerli *et al*., 2007).

The leaves of *Termanalia catappa* demonstrate the ability to prevent infections caused by *A. hydrophila.* In this study, *T. catappa* leaves were added to the fish's basal diet to inhibit the infection caused by the freshwater fish pathogen *A. hydrophila* in *Pocelia sphenops*. Our findings suggest that *T. catappa* leaves may contain several bioactive compounds responsible for their antibacterial effects against *A. hydrophila.*

Materials and Methods

Preparation of plant powder:

The leaves of *T. catappa* were collected from the southern villages of Nagercoil, Kanyakumari district of Tamil Nadu, India. Following collection, the leaves underwent a thorough washing with distilled water. Subsequently, they were shade dried at room temperature. Once dried, the leaves were finely crushed into a powder and passed through an 80-mesh sieve. Finally, the powdered

Table 1: Formulation of experimental diet with *T. catappa* leaf powder (CD: Control Diet, AD1 to AD5: Almond Diet 1 to Almond Diet 5)

Ingredient	Composition	Diet (g /100 g)					
		CD	AD1	AD2	AD3	AD4	AD5
Fish meal	Animal protein	30	30	30	30	30	30
Groundnut oil cake	Plant protein	30	30	30	30	30	30
Rice bran	Carbohydrate	20	20	20	20	20	20
Tapioca powder	Binder	20	20	20	20	20	20
T. catappa leaf powder	Supplement	-	10	15	20	25	30
Total		100	110	115	120	125	130

leaves were stored at room temperature for future use.

Diet preparation:

The basal diet was formulated following the methodology outlined by Giri *et al*. (2012). The ingredients for basal diet were taken in appropriate quantity and were blended in a mixture and cooked and then pelletized, air dried and sieved into appropriate pellets, and were stored at -20º C for further use. The prepared basal diet was used as the control diet. Appropriate quantity of basal diet was supplemented with prepared *T. catappa* leaf powder at 5 different concentrations – AD1-AD5 (Table 1).

Experimental setup:

P. sphenops weighing approximately 2.85 ± 0.06 g were purchased from an aquarium shop located in Nagercoil, Kanyakumari, Tamil Nadu, India. They were carefully transported alive to the laboratory in oxygenated plastic bags. Upon arrival, the fish were acclimatized in four food-grade plastic tubs, each with a capacity of 50 L, for a duration of two weeks under controlled laboratory conditions. Throughout this period, basic water quality parameters were diligently maintained. The dissolved oxygen concentration ranged between 5.2 to 7.2 mg/l^{-1}, measured using the Winkler's method. The pH of the water was maintained within the range of 5.5 to 7.1, and the temperature was kept stable at 22 ± 2ºC. After the two-week acclimatization period, the fish were randomly assigned to six experimental groups. Each experimental group consisted of ten fishes and grown in individual 50 L plastic tubs.

Feeding of herbal diet:

The grouped fishes were fed a diet supplemented with *T. catappa* leaf powder (AD1, AD2, AD3, AD4, AD5) for a period of 30 days. Control (CD) diet was not supplemented with *T. catappa* leaf powder. Feeding to all fish groups involved providing the diet at 3% of the fish's body weight, administered twice daily.

Growth performance parameters:

The fish weight was recorded at the beginning and end of the test. The total weight of fish in each group was measured, and then divided by the number of fish in that group to calculate the average body weight of the fish in each group. Growth parameters were evaluated using the following formulas (Mir Ishfaq Nazir *et al*., 2017):

$$\text{Weight gain} = \text{FBW} - \text{IBW}$$

Where, FBW: Final Body Weight, IBW: Initial Body Weight.

$$\text{SGR (\%)} = \frac{\ln(\text{FBW}) - \ln(\text{IBW})}{(\text{N})} \times 100$$

Where, FBW = Final Body Weight, IBW = Initial

Table 2: Growth parameters of *P. sphenops* in different diet concentrations of *T. catappa*

Diet	IBW (g)	FBW (g)	WG (g)	SGR (%)
CD	2.82 ± 0.12	3.72 ± 0.14	0.90 ± 0.02	0.92
AD1	2.88 ± 0.14	3.83 ± 0.11	0.95 ± 0.03	0.95
AD2	2.84 ± 0.13	3.88 ± 0.12	1.04 ± 0.01	1.04
AD3	2.76 ± 0.11	3.89 ± 0.12	1.13 ± 0.01	1.14
AD4	2.92 ± 0.15	4.13 ± 0.13	1.21 ± 0.02	1.15
AD5	2.85 ± 0.12	4.12 ± 0.10	1.27 ± 0.02	1.22

Data expressed as Mean ± SD, n=10. CD: Control Diet, AD1 to AD5: Almond Diet 1 to Almond Diet5, IBW: Initial Body Weight, FBW: Final Body Weight, WG: Weight Gain, SGR: Specific Growth Rate.

Body Weight, N = Number of days, ln = Natural log.

Preparation of bacteria for challenge test:

The bacteria *A. hydrophila* were isolated from the stock culture and cultured on tryptone soy agar (TSA) using the streak plate method. The plates were then incubated at 37ºC for 24 h. After incubation, colony-forming units of *A. hydrophila* were transferred to 5 ml of tryptic soy broth and placed in a shaker for 24 h at 37ºC. Following incubation, 50 µl of *A. hydrophila* suspension (with a concentration of 1 x 10^6 cells ml^{-1}) was taken for the challenge test.

Challenge test:

After the 30-day feeding period, the fishes from each diet concentration, including the control diet, were intraperitoneally injected with 50 µl of *A. hydrophila* suspension (with a concentration of 1 x 10^6 cells ml^{-1}). Subsequently, the fishes were observed for a duration of 10 days. Abnormal clinical signs were noted, and the survival rate (Opasola *et al.,* 2013) of the fishes was calculated using the formula:

Survival rate (%) = (Number of live fish/Number of fish stocked) x 100

Histopathological analysis:

Fragments of gills from *P. sphenops* exposed to different diet concentrations during the challenge test were fixed in a 10% buffered formalin solution. The organs were subsequently dehydrated in an alcohol solution and cleared in xylene. They were then embedded in paraffin wax at 60°C, and cross-sections of 5 µm thickness were prepared. These sections were stained with hematoxylin and eosin (HE). The slides were mounted and analyzed using a contrast microscope. The histopathological changes in the gills were assessed based on the severity of lesions observed.

Results and Discussion

Growth parameter of P. sphenops:

The effects of *T. catappa* leaves on the growth of *P. sphenops* are shown in Table 2 and Figure 1. The weight gain was higher in AD5 (1.27 ± 0.02 g) and AD4 (1.21 ± 0.02 g) when compared with the weight gain in CD (0.90 ± 0.02 g). The AD1 (0.95 ± 0.03 g) showed higher weight gain than the weight gain of CD (0.90 ± 0.02 g). AD2 (1.04 ± 0.01 g) and AD3 (1.13 ± 0.01 g) showed higher weight gain than the weight gain of CD (0.90 ± 0.02 g) and AD1 (0.95 ± 0.03 g). The specific growth rate (SGR) was higher AD5 (1.22 %) than SGR recorded in other diet groups. The SGR of AD4 and AD3 diet groups were recorded as (1.15 %) and (1.14 %). The control diet showed lowest SGR of 0.92 % than the

Fig. 1: Growth parameters of *P. sphenops* in different diet concentrations of *T. catappa*.

Table 3: Survival rate of *P. sphenops* in different *T. catappa* diet concentrations infected with *A. hydrophila*

Diet	IBW (g)	FBW (g)	WG (g)	Survival (%)
CD	3.72 ± 0.14	3.83 ± 0.11	0.11 ± 0.03	0
AD1	3.83 ± 0.11	3.96 ± 0.14	0.13 ± 0.03	0
AD2	3.88 ± 0.12	4.02 ± 0.15	0.14 ± 0.03	10
AD3	3.89 ± 0.12	4.09 ± 0.14	0.20 ± 0.02	30
AD4	4.13 ± 0.13	4.39 ± 0.10	0.26 ± 0.03	60
AD5	4.12 ± 0.10	4.42 ± 0.12	0.30 ± 0.02	80

Data expressed as Mean ± SD, n=10. CD: Control diet, AD1 to AD5: Almond Diet 1 to Almond Diet 5, IBW: Initial Body Weight, FBW: Final Body Weight, WG: Weight Gain

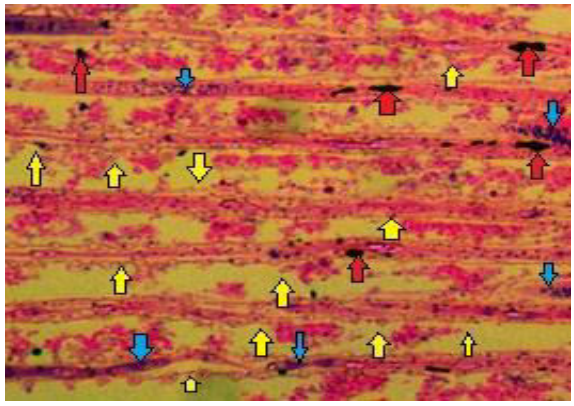
SGR of AD1 (0.95 %), AD2 (1.04 %). In the previous findings by Mir *et al.* (2017), the T6 diet group exhibited the highest weight gain percentage (95.84% ± 1.91) and specific growth rate (SGR) (1.12 ± 0.16) when challenged with *A. hydrophila*. Sukumaran *et al.* (2016) reported that the Ginger powder diet (G8) resulted in higher SGR (3.02 ± 0.06) compared to the control basal diet.

Challenge test:

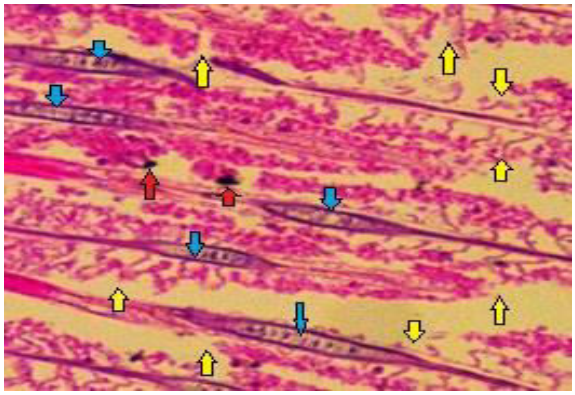
The dietary supplementation of *T. catappa* leaves protected *P. sphenops* against *A. hydrophila* infection. The highest percentage of survival (80 %) was recorded in AD5 diet group and lowest percentage of survival (0 %) was recorded in challenge control diet group and AD1 diet group (Table 3). In the challenge test against the T6 diet group demonstrated the highest survival percentage compared to the control group (Mir *et al.,* 2017). The survival rate was found to be higher in fish fed with moringa supplements against *A. hydrophila* (Gbadamos *et al.,* 2016).

Histopathological analysis:

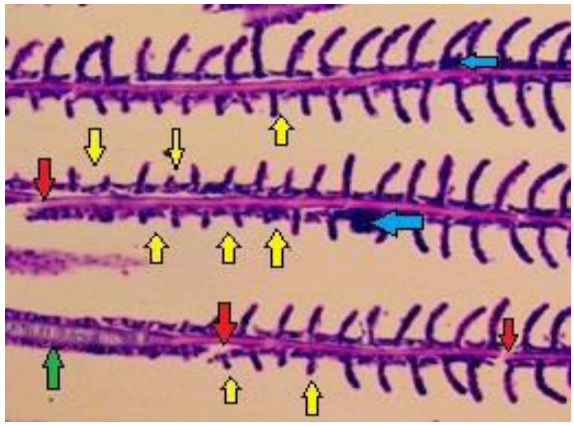
In the histological study, the gill filaments of *P. sphenops* given the CD diet and AD1 were severely damaged. There were several fragmentations and blood congestion in the secondary lamella. *P. sphenops* fed with AD2 diet showed decreased



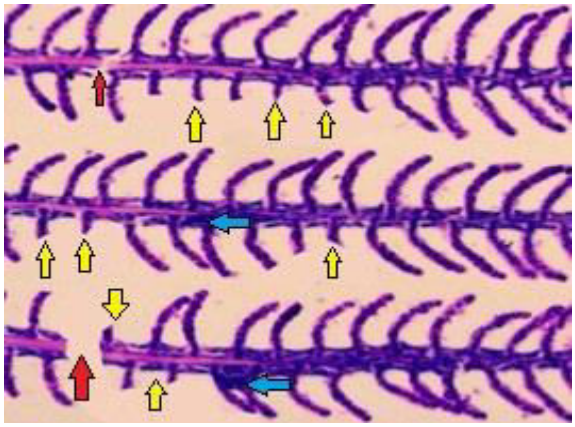
(A) CD (Control diet) Yellow arrow: Fragmentation of secondary lamella, Red arrow: Blood congestion, Blue arrow: Hyperplasia



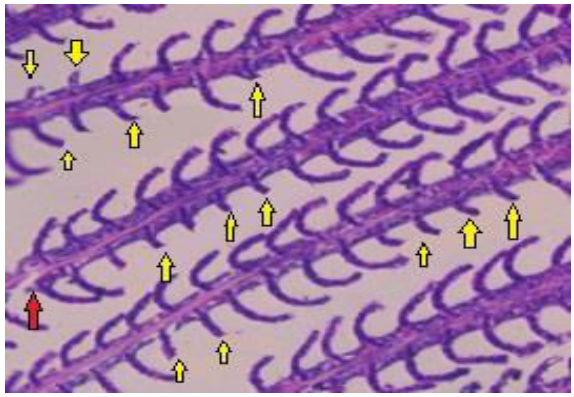
(B) AD1 (Almond Diet 1) Yellow arrow: Fragmentation of secondary lamella, Blue arrow: Swelling of primary lamella, Red arrow: Blood congestion



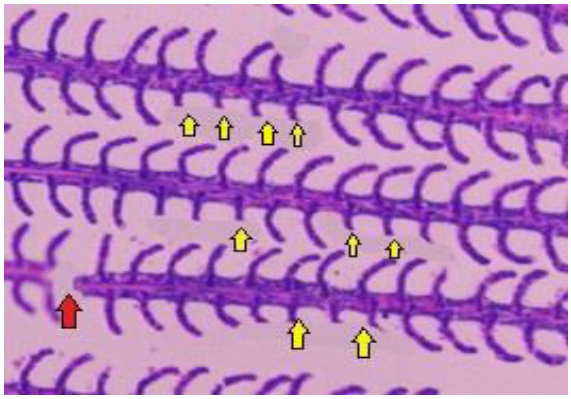
(C) AD2 (Almond Diet 2), Yellow arrow: Shortening of secondary lamella, Red arrow: Fragmentation in primary lamella, Green arrow: Swelling of primary lamella, Blue arrow: Hyperplasia



(D) AD3 (Almond Diet 3), Yellow arrow: Shortening of secondary lamella, Red arrow: Fragmentation in primary lamella, Blue arrow: Hyperplasia



(E) AD4 (Almond Diet 4) Yellow arrow: Shortening of secondary lamella, Red arrow: Fragmentation in primary lamella



(F) AD5 (Almond Diet 5) Yellow arrow: Shortening of secondary lamella, Red arrow: Fragmentation in primary lamella

Fig. 2: Histopathological sections of gills of *A. hydrophila* infected *P. sphenops* fed with *T. catappa* leaves.

fragmentation and hyperplasia compared to CD and AD1. The growth of gill filaments began with the AD3 diet. Primary and secondary lamella showed less fragmentation in the AD4 and AD5 diets.

Thus, histological investigation revealed that the fragmented lamella found in the gills of *P. sphenops* fed CD and AD1 diets infected with *A. hydrophila* improved on AD4 and AD5 diets. In the histological study, the gill filaments of *P. sphenops* given the CD diet and AD1 were severely damaged. The secondary lamella of *P. sphenops* fed CD and AD1 diets showed many fragmentations. Blood congestion and hyperplasia was also observed in CD and AD1 diet group. Swelling of primary lamella was observed in AD1 diet group. *P. sphenops* fed with AD2 diet showed decreased fragmentation and hyperplasia compared to CD and AD1. The development of gill filaments started with the AD3 diet. Improved structure of primary and secondary lamella were observed in the AD4 and AD5 diets. Thus, histological investigation revealed that the fragmented lamella found in the gills of *P. sphenops* fed CD and AD1 diets infected with *A. hydrophila* improved on AD4 and AD5 diets. The histopathological sections of gills of *A. hydrophila* infected *P. sphenops* fed with *T. catappa* leaves are shown in Figure 2 (A, B, C, D, E, F).

Fish afflicted with *A. hydrophila* exhibited a range of detrimental conditions in their gills, including hyperplasia, haemorrhage, necrosis, and congestion. Hyperplasia, characterized by an increase in lamellar epithelial cells, particularly between the primary and secondary lamellae, leads to damage to red blood cells, resulting in oxygen deprivation and eventual death in the fish (Rosidah *et al.*, 2020).

Conclusion

To prevent the infection caused by *A. hydrophila*, *T. catappa* leaves have been identified as an effective measure. In this study, the basal food for *P. sphenops* is supplemented with *T. catappa* leaves to hinder the infection by the freshwater fish pathogen *A. hydrophila*. Our investigation suggested that the antibacterial properties of *T. catappa* leaves against *A. hydrophila* may be attributed to a range of bioactive chemicals.

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