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Achyranthes aspera Extract as feed additives enhances immunological parameters and growth performance in *Pangasius pangasius* against *Pseudomonas fluorescens*

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Abstract

Pangus (*Pangasius pangasius*) has been increasing for its high potential and play vital role in aquaculture in Bangladesh. The effects of dietary *A. aspera* supplementation on innate immunity, growth and disease against *P. fluorescens* infection were evaluated in Pangus (*P. pangasius*). Fish were feeding for 28 days with control diet and three experimental diets containing *A. aspera* herbal diet which were prepared with at 0%, 0.5%, 1% and 1.5% of root extract respectively. Various innate immune parameters examined at 14 and 28 days post-feeding. All four Groups of pangus injected intraperitoneally with *P. fluorescens* (3.2×10^6 CFU ml⁻¹) and after 14 and 28 days of post immunization, bloods sampled collected from live fish with a hypodermic syringe. The present results demonstrated that Dietary supplementation of *A. aspera* (1.5%) showed significantly increased serum antibody titer (3280), phagocytic activity (49%), immune response as both specific (44%) and non-specific (3%), activation of disease resistance and growth factors (41.1%) of *P. pangasius* ($P < 0.05$) compared with the control group throughout the experimental period. The infected pangus and those treated with 1.5% of herbal supplementation feeds exhibited significantly less mortality (25%) indicating highest protection (Relative Percent Survival 70%) from *P. fluorescens* infection than compared to the control group. The results indicate that 1.5% of *A. aspera* herbal supplementation feeds were restored the altered hematological parameters and triggering the innate immune system of pangus against *P. fluorescens*.

Keywords: *Pangasius pangasius*, *Pseudomonas fluorescens*, *Achyranthes aspera*

1. Introduction

In Bangladesh, scientific information about bacterial diseases in fish are in preliminary stage while it is scare in the field of *P. fluorescens* which has been denoted as one of important disease causing agents of both farmed and wild fish. *P. fluorescens* described as the causative agent of Bacterial Hemorrhagic Septicemia disease of pond-cultured fish [1]. Muniruzzaman and Chowdhury (2004) have evaluated sensitivity of certain local medicinal herbs against fish pathogenic *P. fluorescens* isolates and argued that the extract of bulb of *Allium sativum* had significant inhibitory effects. In the recent past, there has been a tremendous increase in the use of plant based health products in developing as well as developed countries resulting in an exponential growth of herbal products globally. One of the many plants used is *A. aspera*. *Achyranthus aspera* Linn. Belongs to the family Amaranthaceae, is an annual, stiff erect or procumbent, annual or perennial herb, 1-2m in height, often with a woody base, commonly found as a weed of waysides, on roadsides [3, 4, 5]. Above study designed to evaluate anti-inflammatory activity of an aqueous extracts of *A. aspera* against *P. fluorescens* [9]. Ethyl acetate extracts of *A. aspera* have proved that to contain anti parasitic activity [7]. They have reported that the ethanol and chloroform extracts of seeds of *A. aspera* shows mild to moderate antibiotic activity against *B. subtilis*, *E. coli* and *P. aeruginosa* [8]. They have studied that extracts of *A. aspera* shows the maximum inhibition of *E. coli* (17 mm) [6].

2. Materials and Methods

2.1 Fishes and management

Pungasius pungasius, pungus (30±6g, N= 48), were obtained from the local fish farm, Jessore (23.17°N 89.20°E), Bangladesh and were transported to the laboratory of the Dept. of Fisheries and Marine Bioscience (FMB), Jessore Science and Technology University (JUST), Jessore, on July 2014 to February 2015. The fish acclimatized for 3 weeks and take care on health condition under close observation. Fish were cultured in indoor glass tanks (100 L). Fishes divided into four groups (25 fishes/group) under two feeding regimes, control: four groups test: four groups. Temperature, pH and TDS (total dissolved solid) ranged from 30±0.7 °C, 6.84±0.08 and 422±0.34 respectively during the experiment. Dissolved oxygen level maintained above 5±0.32 mg/l throughout the experiment. Fishes were provided with normal feed (without herbal extract) at the rate of 4% of their body weight twice a day at morning and evening but first day of their arrival no feed was provided.

2.2 Collection of plant material and preparation of extract (*A. aspera*)

Plant material was collect from local areas of Jessore (*A. aspera* date: 05-05-14). The leaves of *A. aspera* (known value showed in Table 1) were washing thoroughly first with running tap water and then washed with sterile distilled water. Leaves (dried) were pulverize using an electrical grinder and root extract were collected manual pressure by iron grinder and extracted with distilled water at 100 °C for 4 h, centrifuged at 5,000 rpm for 15 min, and filtered using what man no.1 filter paper. The residues obtained after evaporation of ethanol kept in sterilized screw cap glass container and stored at -20 °C until analysis.

Table 1: The amount of nutritional, microbiological and toxicological evaluation of *A. aspera*

Components	Amount
Moisture	4.05%
Proteins	20.54%
Fats	0.903%
Ash	20.25%
Carbohydrates	54.26%
Energy	294 Kcal
Phosphorus	1447.5mg/kg
Microbiological	Nutritive supplement in blood
Toxicological	Good effect on general health (weight)

Reference: [22].

2.3 Herbal diet preparation

The experimental diet was prepared by mixing locally available mega feed that proximately contains protein: 34%, crude fiber: 6%, crude ash: 18%, moisture: 11%, lipid: 6%, fat: 3% (source: Spectra Fish Feed Company Ltd.). At first mega feed were grinded by a grinder and mixed with *A. aspera* extract. All the ingredients were mixed thoroughly by adding water and pelletized manually and after then all pelleted feed kept under sun light for drying. Four different experimental pellet diets were prepared which contained four different percentages of *A. aspera* such as 0%, 0.5%, 1.0% and 1.5% [22]. The prepared feed was then sun dried under sterile condition for 3 days and stored in a glass airtight container.

2.4 *Pseudomonas fluorescens* isolation

P. fluorescens strains were initially isolated from dropsy and septicemia pungus, which used in this study. The disease fish collected from the hatchery in Jessore, during the winter season. Those strains since their isolation being maintained in laboratory, by repeated culture in selective agar media (*Pseudomonas* agar media). Total stocks were growing in pseudomonas agar media for 24 hrs at 37 °C over night and kept in -20 °C until use. The subculture was taken and centrifuged (5000 rpm for 12 min), after centrifugation, the supernatant was discarded and the pellet was re-suspended in sterile phosphate buffer saline (PBS). The culture adjusted at 3.2×10^6 colony forming units (CFU) ml⁻¹ and incubated at 37°C for 24 hrs. The bacteria confirmed by the following characterization.

Table 2: Identifying characteristics of fish pathogenic strain *P. fluorescens*

S. No	Character	Results
1	Colony shape	Round
2	Colony size	Medium
3	Colony color	Yellowish
4	Gram stain	-
5	Shape	Rod
6	Oxidase	+
7	Polar flagella	+
8	Catalase	+
9	O-F test	Oxidative
10	Motility	+
11	Methyl-Red test	-
12	Growth at Tween20	+
13	Growth at 4oC	-
14	Growth at 37oC	+
15	Growth in 0% NaCl	+

Note: + = positive reaction; - = negative reaction;

2.5 Experimental Design

The experiment performed in 120 L rectangular glass aquarium in the laboratory. The fishes divided into four groups of treatment (0%, 0.5%, 1.0%, and 1.5%) of 10 fishes each in triplicate. Fishes were provided with adequate aeration and fed at the rate of 3% (morning) and 2% (evening) of body weight of fish twice a day with the respective diets till the end of experiment (28 days). Two fishes randomly separated from each experimental aquarium maintaining every week to collect blood and analysis for the non-specific immunological assays. On 28th day of feeding, all groups injected intra-peritoneal (i.p.) with 25µl PBS containing *P. fluorescens* at 3.5×10^7 CFU ml⁻¹ for analyzing cumulative mortality.

2.6 Growth Performance

The growth performance of weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) were determine according to Choudhury *et al.*, (2005).

$$\text{Percentage of weight (g) gain} = \frac{\text{Final weight (g)} - \text{Initial weight(g)}}{\text{Initial ewight (g)}} \times 100$$

$$\text{Percentage of specific growth rate (SGR)} = \frac{\text{Final weight (g)} - \text{Initial weight(g)}}{\text{Initial ewight (g)}} \times 100$$

$$\text{Feed conversion ratio} = \frac{\text{Feed intake per body weight}}{\text{Weight gain}}$$

2.7 Bleeding and serum separation (specific immune response assay)

Two fishes selected randomly from each group for blood collection. Blood were collected from caudal vein of the fishes from four groups separately with the help of sterilized hypodermal syringe containing EDTA (Ethylene-Diamine-Tetra-Acetic) as an anticoagulant and all collected blood kept in 1.5 ml eppendorfs. For separating the serum from the blood, the eppendorfs with blood sample placed into a centrifuge machine at 120000 rpm for 7 min. For each group of three cultures plates were prepared with 0%, 0.5%, 1.0% and 1.5% of diet. Bacterial stock solution were serial diluted for 10 times and 10^{-4} , 10^{-5} , 10^{-6} concentration were selected from further usage. Then 25 μ l volume for each (10^{-4} , 10^{-5} , 10^{-6}) diluted solution was mixed with 25 μ l separated serum from four different groups of fishes then spread in different culture plates and finally all plates were placed in a incubator at 37°C for 24 hrs. After 24 hrs, all plates observed.

2.8 Immune response assay

The phagocytic activity and serum agglutination titer quantified by following the modified method of (Swan *et al.*, 2010)

2.9 Mucus collection and bacteria culture (non-specific immune response assay)

Mucus collected by scraping the body surface and gill of fishes with a scalpel from four groups (0%, 0.5%, 1.0%, and 1.5%) and collected mucus kept in four Eppendorf's separately. Same like, as serum and bacteria culture three culture plates for each group were prepared as followed by disc diffusion method. 25- μ l of mucus from the each group mixed with same volume of three different diluted bacterial solutions (10^{-4} , 10^{-5} , and 10^{-6}) and finally all plates placed in an incubator at 37 °C for 24 hrs. After 24 hrs, all plates observed.

2.10 Phagocytic activity

For observing the phagocytic activity assay, 25- μ l blood cell suspension from pungas and 25- μ l bacterial solution in PBS previously fixed with glutaraldehyde placed on a cover slip. After 30 min, cover slip carefully washed with PBS then air-dried and fixed with methanol and after that stained with Giemsa. The engulfed fish blood cell was determined by using photographic microscope (Axiacom ERc 5s with Axio Vision driver Carl Zeiss, Germany).

2.11 Serum agglutination titer assay

At day 7, 14, 21 and 28 of the experiment blood sample were

collected from each group of fish. Serum samples were collect by following centrifugation. Isolated bacterial cell suspensions centrifuged in 7000 rpm for 12 min and supernatant discarded. The resulting plates washed twice with PBS solution and then plates re-suspended with PBS. Starting with a dilution of 1:10 (10 μ l serum and 90 μ l PBS) two-fold serial serum dilutions were made in 96-well round bottom micro titer plates by adding 25 μ l of diluted serum into the remaining wells plate with 25 μ l of bacterial cell suspension was added to each well. The plate were covered with plastic film and incubated at 4 °C for 2 hrs and 24 hrs incubated at 25 °C. Result of agglutination titer was determined by using multi-scanner.

2.12 Challenge test

For the challenge test, virulent *P. fluorescens stains* prepared from the serial dilution. Two days after the last bleeding, the fishes from each group injected intra-peritoneal with 1ml of 24 hours cultured *P. fluorescens*, which contained 3.2×10^{-6} CFU ml⁻¹ challenge stain. The clinical signs and mortality recorded up to 28 days of post challenge. The cumulative mortality calculated by following Amend (1981) and Relative Percent Survival (RPS) calculated as follows

$$\text{RPS} = 1 - \frac{(\% \text{ Mortality in treated group})}{(\% \text{ Mortality in control group})} \times 100$$

2.13 Statistical analysis

Values for each parameter measured expressed at the arithmetic mean \pm standard error (SE). Effects of herbal diets on growth performance, hematological and immunological parameters tested using one-way ANOVA and the mean values compared by using Duncan's multiple range tests at 0.5% level of significance (Zar, 1984)

3. Results

3.1 Disease resistance (Challenge test)

The total numbers of fish samples in four-treatment groups were 48 but there is no great variation between the individual fish. From the data (table 3), it is evident that the highest mortality rate was in control group as compared with the others. The infected fishes are low in 1.5% doses compared with others, resulting low level of mortality. All the treatments are always present in similar doses (3.2×10^{-6} CFU ml⁻¹). The cumulative mortality was lowest (Fig. 1 and 2) 25% when fed with 1.5% supplemented diet compared with control (83.33%) and other dose diets, which were 41.67%, and 75% in case of 1%, 0.5% supplemented diets respectively. In this study, 1.5% supplemented diet showed 75% survivability and 70% RPS (related percentage survival) which was higher than other treatments (Table 3).

Table 3: Challenge treatment of *A. aspera* against *P. fluorescens* in pangus at 28th days of the experiments.

Treatment	Challenge dose (CFU ml ⁻¹)	Total fish	No. of infected fish	No. of death fish	Mortality (%)	Survivability (%)	RPS (%)
Control	3.2×10^{-6}	12	11	10	83.33	16.67	0
0.5%	3.2×10^{-6}	12	10	9	75	25	10
1.0%	3.2×10^{-6}	12	10	5	41.67	58.33	50
1.5%	3.2×10^{-6}	12	7	3	25	75	70

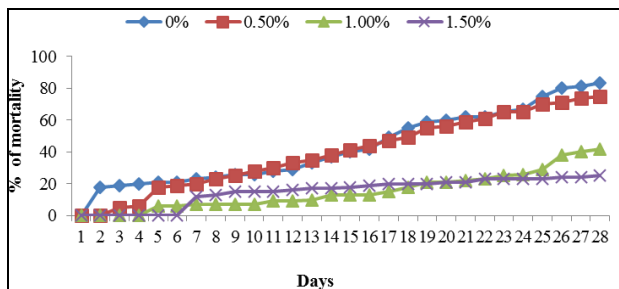


Fig 1: The cumulative mortality of *P. pangasius* fed with different doses of *A. aspera* supplemented diets against *P. fluorescens*. [* indicates relatively significant ($P<0.05$)]

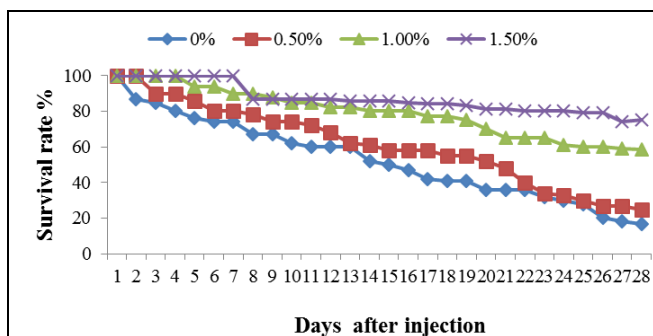


Fig 2: Survival rate of *P. pangasius* fed with different doses of *A. aspera* supplemented diets against *P. fluorescens*. [* indicates relatively significant ($P<0.05$)]

3.2 Serum agglutination titer assay

Measurement of Serum agglutination titer assay, fish diet was continues 28 days until analysis. After 4 weeks of feeding, fish immunized spleen and blood sampled on weekly intervals for four times after immunization. *Achyranthes* has significantly ($P<0.05$) enhanced the BSA-specific antibody titers than the untreated control group throughout the study period. The efficiency of antigen clearance also enhanced in *P. pangasius* treated with *Achyranthes*. Serum agglutination titer assay (Table 4) completed on 14th day and 28th day of the experimental period. 1.5% of *A. aspera* added diet fed fishes and highest diluted serum (3280) showed positive agglutination ($0.29\pm.02$; $0.06\pm.01$) response (Fig. 3).

Table 4: Different immune parameters of Pangus at 14 days and 28 days of the experiment.

Immune parameters	Control		0.5%		1%		1.5%	
	14 days	28 days	14 days	28 days	14 days	28 days	14 days	28 days
Serum agglutination	0.65	0.38	0.57	0.27	0.42	0.12	0.29	0.06

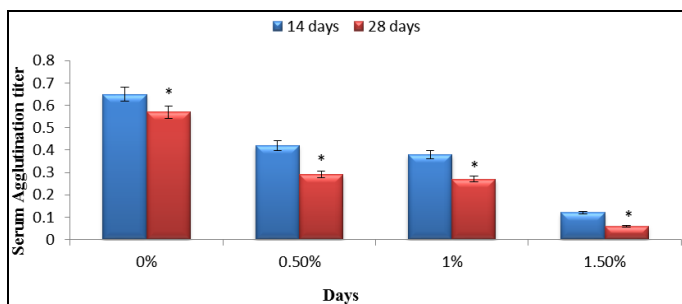


Fig 3: Serum agglutination titer assay of *P. pangasius* fed with different doses of *A. aspera* supplemented diets against *P. fluorescens*. [* indicates relatively significant ($P<0.05$)]

3.3 Phagocytic activity

The phagocytic activity was significantly ($P<0.05$) higher in test groups of fish than the control group on days 21 and 28. These results showed the immune-stimulatory activity of the prepared diet containing root extract of *A. aspera*. Phagocytic activity did not significantly enhance with 0.5%, 1.0% and 1.5% enriched diet on first week against *P. fluorescens*. However, with 1.0% and 1.5% doses the activity significantly increased on week 3 and 4 but not with 0.5% and 0.5% doses of supplemented diet, as compared with the control (Fig. 4).

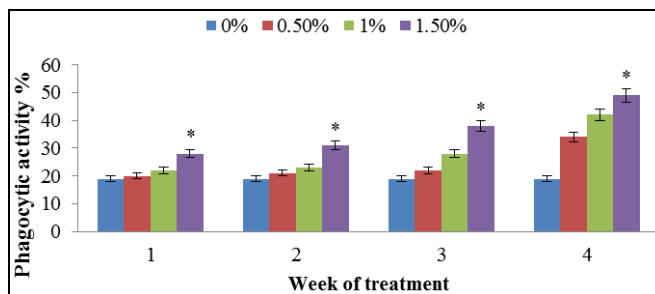


Fig 4: Phagocytic activity (%) of *Pangasius pangasius* fed with different doses of *A. aspera* supplemented diets against *P. fluorescens*. [* indicates relatively significant ($P<0.05$)]

3.4 Specific immune response assay (Serum, bacteria culture)

Fishes feeding with different doses of *A. aspera* (0.5%, 1.0%, and 1.5%) did not significantly change immune response on first week. Immune response level significantly increased with 1% and 1.5% supplemented diets on week 3 and 4 (Fig. 5). However, immune response level did not significantly change in control.

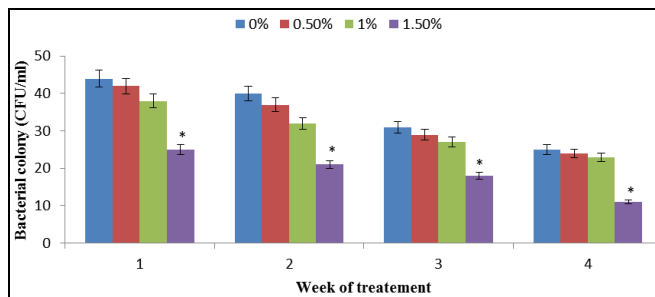


Fig 5: Bactericidal activity of serum of *P. pangasius* fed with different doses of *A. aspera* supplemented diets against *P. fluorescens*. [* indicates relatively significant ($P<0.05$)]

3.5 Non-specific immune response (Mucus, bacteria culture)

Selected bacterial plate was ready for measured of non-specific immune response by serial dilution. Here, only Nutrient and BHI media used to determine the results by spreading the mucus on the agar plate. Measurements performed at 30° Cover night. Fish feeding with 0.5% and 1.0% *A. aspera* enriched diet did not significantly enhance the immune response at 28th day in pangus against *P. fluorescens* compared to control diet (0%). Fish fed with 1.5% *A. aspera* enriched diet showed significantly enhanced immune response (Fig. 6) from week 1 to 4 compared to the control.

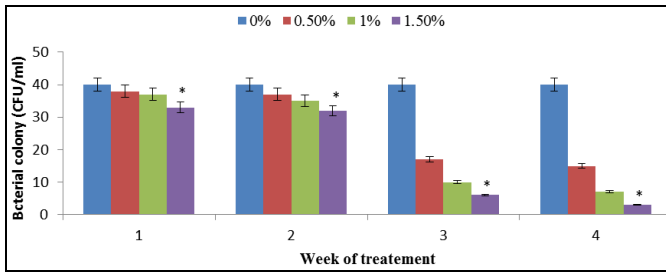


Fig 6: Bactericidal activity of mucus of pangus fed with different doses of *A. aspera* extract supplemented diets against *P. fluorescens* [* indicates relatively significant ($P<0.05$)]

Table 5: Growth parameters of pangus fed with different doses of *A. aspera* supplemented diets against *P. fluorescens*.

Growth parameters	Doses	Week-1	Week-2	Week-3	Week-4
WG	0%	32.02±1.2	32.09±1.3	33.03±1.5	35.05±1.5
	0.5%	29.40±1.4	30.1±1.6	34.33±1.7	36.13±1.7
	1.0%	32.12±1.5	33±1.7	35.56±1.5	37.21±1.5
	1.5%	34±1.4	35.88±1.4	36.12±1.7	41.1±1.7
SGR	0%	1.12±0.14	1.00±0.6	1.69±0.4	2.00±0.14
	0.5%	1.12±0.2	1.31±0.4	1.90±0.3	2.12±0.31
	1.0%	1.01±0.4	1.12±0.23	1.45±0.41	1.16±0.4
	1.5%	1.14±0.3	1.70±0.6	2.20±0.5	2.45±0.5
FCR	0%	1.5±0.2	1.6±0.1	1.7±0.3	1.7±0.3
	0.5%	1.5±0.1	1.6±0.2	1.6±0.3	1.6±0.3
	1.0%	1.4±0.2	1.5±0.3	1.6±0.1	1.6±0.1
	1.5%	1.2±0.3	1.3±0.2	1.3±0.3	1.3±0.3

Note: WG = Weight gain, SGR = Specific growth rate, FCR = Food conversion ratio. [* indicates relatively significant ($P<0.05$)]

5. Discussion

The aim of this study was to find a natural source of antimicrobial substances to replace antibiotics for the treatment of *P. fluorescens* infection in fish aquaculture. The *P. fluorescens* used in this study that was isolated from naturally occurring pangus, which was exhibiting MAS (motile *P. fluorescens*) from the hatchery complex of Jessore (Ma Fatema Hatchery). However, *A. aspera* has shown to boost immune defense property in Pangus but the use of *A. aspera* in aquatic species for their disease treatment especially for stimulating the immune responses has not established yet. The mortality showed that *A. hydrophila* decreases the cumulative mortality with increasing concentration of *A. aspera*. It showed the cumulative mortality rate was 50% for D4, 40% for D1, 35% for D2, and 15% for D3. In this present study showed decrease in mortality rate with *A. aspera* diet. Injection of *A. hydrophila* was similar in *Labeo rohita* fed with diet containing *Achyranthes aspera* [10]. *Achyranthes aspera* incorporated in artificial fish diet and fed to Catla against *A. hydrophila* [11].

In this present study showed, the treatment groups mortality were decreases at 1% and 1.5% of *A. aspera* formulated diet feeding and the mortality rate was 41.67% and 25% respectively. The study have proved that herbal diet of *A. aspera* supplements enhance the survivability of Pangus than the control against *P. fluorescens*.

Achyranthes has significantly ($P<0.05$) enhanced the BSA-specific antibody titers than the untreated control group throughout the study period. The efficiency of antigen clearance also enhanced in Catla treated with *Achyranthes* [11]. Hemagglutination antibody titers were significantly higher in the test group of fishes compared with the control group [13]. In this present study, it was demonstrated that the highest diluted serum (5, 00,143) showed positive agglutination (0.29±0.08; 0.06±0.07) treated by 1.5% than the control (0.65±0.08.02; 0.06±0.07).

The present study has revealed that the phagocytic activity

3.6 Growth performance

All Pangus fed with different percentages of *A. aspera* supplemented diet (0.5%, 1% and 1.5%) showed significant growth as compared to the control group. However, with 0% and 0.5% *A. aspera* added diets the specific growth rate (SGR), feed conversion ratio (FCR) did not significantly increased (Table 5).

has increased when fed with 1% and 1.5% *A. aspera* supplemented diets on week 3 and 4 but it did not showed any response against the pathogen (*P. fluorescent*) on the first week. The present study findings are in the line with the report in goldfish against *A. hydrophila* fed with the diet containing herbal extract supplementation [12]; microbial levan at 1.25% used as dietary immune-stimulant for *L. rohita* juveniles [14]. An increased in immunomodulatory activity of *Solanum trilobatum* leaf extracts in *Oreochromis mossambicus* [14].

The present study showed bactericidal activity of serum significantly increased in 1% and 1.5% treated by *A. aspera* diet on week 3 and 4 respectively compared to the control group. Similarly, work on *Lactuca indica* extract as feed additive enhances immunological parameters and disease resistance in *Epinephelus bruneus* to *Streptococcus iniae* [14]. Significantly, leaf extract of *Ocimum sanctum* increased the immune-stimulatory effect of *Oreochromis mossambicus* [16]. Same like as bactericidal activity of serum and the bactericidal activity of mucus also showed efficient result. Fishes fed with 1% and 1.5% *A. aspera* diet dramatically enhanced specific and non-specific immune response than the control group. Dietary intake of *O. sanctum* also enhanced the specific and non-specific immune responses against *A. hydrophila* [16]. Oral administration of *Eclipta Alba* aqueous leaf extract enhances the nonspecific immune responses and disease resistance of *Oreochromis mossambicus* [17]. Chinese herbs (*Lonicera japonica* and *Ganoderma lucidum*) enhance non-specific immune response of *Oreochromis niloticus*, and protection against *A. hydrophila* [18].

The specific growth rate exhibited an increased trend in all the four experimental groups, however it was significantly higher in 1.5% *A. aspera* added diet fed fishes. Thus, it evident that dietary supplementation of *A. aspera* acted as growth promoter. They have studied on Thai pangus polyculture with carps has been increasing for its high potential, however very few attempts were made to compare its growth using different

types of feed ^[20]. *W. somnifera* root powder have a stimulatory effect on immunological parameters and increases disease resistance in *L. rohita* fingerlings against *A. hydrophila* infection ^[19]. This study result shown that herbal growth promoter effects of feed additive in fish meal on the performance of *Oreochromis niloticus* ^[21].

6. Conclusion

The overall outcomes of the result shown that 1.5% of *A. aspera* extract induced highest positive response against *P. fluorescens* and exhibited the more optimum features in immunological parameters to a significant level. Hence, it can be stated that the inclusion of *A. aspera* extract at a rate of 1.5% in the fish feed would be ideal to elicit immunity and disease resistance (survival rate 75%) in *P. pangasius* against *P. fluorescens* infection. Taken together, these data suggest *A. aspera* to have potent anti-stimulant property.

7. Acknowledgement

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