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Immunostumilant activity of herbal feed on *Aeromonas hydrophila* infection on snakehead fish *Channa punctatus* (Bloch,1973)

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Abstract

The present study has been carried out the effect of medicinal plants like *Cynodon dactylon* and *Cassia auriculata* on different concentrations of formulated diet against *Aeromonas hydrophila* infection on *Channa punctatus.* The parameters such as, anti-protease activity, total heterotrophic count and antioxidant enzymes were studied. The results of feeding the fishes with experimental diet has significantly (p<0.05) enhanced the serum anti protease and total heterotrophic count than fishes fed with control diet.

Keywords: *Channa punctatus*, *Cynodon dactylon, Cassia auriculata, Aeromonas hydrophila,* Antiprotease activity, Total Hetertrophic count; Antioxidant enzyme.

Introduction

Aquaculture fish production has increased significantly over the past few decades, which has led to intensive fish culture practices where stressors like overcrowding, transport, handling, grading and poor water quality are common (Christybapita *et al.,* 2007). These stress factors can result in immune depression and outbreaks of infections. The use of compounds with immune stimulant and/or antioxidant effects as dietary supplement can improve the innate defense on animals providing resistance to pathogens during periods of high stress (Faggio *et al.,* 2015). Among all other bacteria, *Aeromonas* and *Pseudomonas* are the major bacterial fish pathogens which widely distributed in aquatic organisms in nature (Banu and Islam *et al*.,1996) With the outbreak of EUS in 1988 *Channa* sp and many other species were severely affected (Barua *et al.,* 1989).

A. hydrophila is a widespread, opportunities pathogen, causes high mortality of cultured and feral (McDaniel,1979). It is the causative agent of the disease known as'haemorrhagic septicemia','ulcer disease', or 'red-sore disease'. *A.hydrophila* is generally found in the gastrointestinal tract of fish is considered an opportunistic pathogen. Most of the bacteria, which are termed 'opportunistic' usually, do not cause disease unless other factors are involved. *A. hydrophila* is always capable of producing disease if given the chance (Adanir and Turutoglu,2007). Medicinal plant extract has been tested in good results for the growth of fishes. Hence, the present study focuses to assess the effects of herbal feed of *C. auriculata* (L.) and *C. dactylon* (L.) on innate immunity and disease resistance of *C. punctatus*.

Materials and Methods

Acclimatization of laboratory condition

Freshwater fish of *C. punctatus* were obtained from Periyakulam pond, Sivakasi, Virudhunagar, Tamilnadu, India. The fish were brought to the laboratory in plastic bags in oxygenated habitat water and acclimatized for 30 days in disinfected 1000 L FRP (Fiberglass Reinforced Plastics) tanks. During the acclimatization, the fingerlings were fed with normal diet. Healthy and disease, free fishes weighing average body weight of 25-55 g were selected for further experiments.

Feed preparation

Fresh leaves of *Cynodon dactylon* and *Cassia auriculata* were collected from agriculture field area, Sivakasi. Fresh leaves of *C. dactylon* and *Cassia auriculata* and were collected, washed, shed dried and ground into fine powders and used for preparation of feed preparation method of according to Karpagam and Krishnaveni, (2014) method.

Preparation of test diets

Fish feed was prepared by adding equal proportions of fish meal, soya bean powder, wheat flour and Groundnut oil cake in the ratio of 4:2:2:1 and rice flour as a binder. These substances were mixed thoroughly with hot water and it was steamed for 25-30 minutes and then cooled at room temperature for 30 minutes and the multi vitamin and mineral tablets were added. Six experimental diets were prepared by adding 1 and 3 grams of plant powders to the semi moist dough and the feed without plant powder was kept as control.

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Table-1: Preparation and feed composition of experimental diets

Ingredients (g/100gms)	Control	*Cassia auriculata* (A)		*Cynodon dactylon* (B)		Mixture (A) and (B) 1:1(C)	
		A1	A2	B1	B2	C1	C2
Fish meal	44	44	44	44	44	44	44
Soyabean meal	20	20	20	20	20	20	20
Groundnut oil cake	20	20	20	20	20	20	20
Wheat flour	10	10	10	10	10	10	10
Rice bran	4	4	4	4	4	4	4
Fish oil	1	1	1	1	1	1	1
Vitamin	1	1	1	1	1	1	1
Herbal immunostimulant (mg)	0	1	3	1	3	1	3

Bacterial strain

Aeromonas hydrophila is belonging to the family, Aeromonadaceae. A pure virulent strain of *A. hydrophila* received from microbiology department, (ANJA College) was maintained at 4°C. From this culture, sub-cultures were maintained on Nutrient Agar (NA) slants (Hi-media, Mumbai) at 5°C. A stock culture in Nutrient Broth (NB) was also maintained at -20°C with 0.85% NaCl (w/v) and 20% (v/v) glycerol to provide stable inoculated throughout the study period (Yadav *et al.,*1992).

Experimental diets and feeding observation

The selected experimental animals of healthy and disease-free fishes of *C. punctatus* weighing average body weight of 25-55gms for acclimatization for 20days. Bacterial suspension of *A. hydrophila* (0.2ml of 10^5CFU/ml) induced in healthy fishes of *C. punctatus*. After the few day, virulent strains of *A. hydrophila* was produced symptom of disease in experimental fishes. But formed in experimental animal fishes fed on experimental diet and control diet for twice a day for 35 days. Each treatment was performed in triplicate. Nutrients composition of trails was showed in Table -1. At the end experimental period, each experimental fish was taken from each tank and the blood was collected for the analysis. After the preliminary investigations like length and weight of the fish, the fishes were placed on a tray and a towel was used to clean the body to avoid the mixture of blood and water. Blood samples were obtained from the caudal fin with the aid of a clinical disposable plastic syringe. The needle was inserted at right angle to the vertebral column of the fish and was gently aspirated during penetration. The needle was pushed gently down until blood started to enter the needle. The blood was drawn gently until about 3 cm^3 of blood was obtained. Thereafter, the needle was withdrawn and the blood immediately transferred into a heparinized plastic tube. The sample was gently but thoroughly mixed to avoid coagulation. Blood samples were used for the measurement of haemoglobin concentration, red blood cell count, white blood cell count (Kesena and Eseoghene, 2016).

Innate Immune Parameters

Antiprotease Activity

The serum anti-trypsin activity was measured by the established methods of Ellis (1987) and Lange *et al*., (2001). Thus, 20 µl of standard trypsin solution (Sigma-Aldrich, 5 mg/ ml) was incubated with 20 µl of serum for 10 min at 22ºC. Subsequently, 200 µl of 0.1 M PBS (PH 7.2) and 250 µl of 2% azocasein solution (20 mg ml-1 PBS) were added and incubated for 1 h at 22ºC. The reaction was then ended with the addition of 500 µl of 10 % (v/v) trichloro acetic acid (TCA) and incubated for 30 min at 22ºC. The mixture was centrifuged at 6000 x g for 5 min and 100 µl of the supernatant was transferred to a flat-bottomed 96 well plates containing 100µl of 1 *N* NaOH /well. The absorbance was read in the spectrophotometer at 410 nm, and the percentage inhibition of trypsin activity was calculated by comparing with a 100% control sample, in which the buffer replaced the serum. For a negative control, the buffer replaced both serum and trypsin (Abbas and Awad,2016). The percentage of trypsin inhibition was calculated as described by Rao and Chakrabarti (2004).

$$\% \text{ of Trypsin inhibition} = \frac{\text{Trypsin blank OD (A1)} - \text{Sample OD (A2)}}{\text{Trypsin blank OD (A1)}} \times 100$$

Enumeration of Total Heterotrophic Count (THC)

One gram of sample from intestine were taken from the diseased fish with moderate lesions, wound and septicemia using a sterile scalpel under aseptic condition. A small piece of muscle was homogenized with sterile distilled water and centrifuged at 1000 rpm for 10 minutes. One ml of the supernatant was serially diluted up to 10-9 dilution. 1ml was taken from each dilution and pour plate technique was carried out for the enumeration of total heterotrophic bacterial count using sterile nutrient agar for bacterial growth. The bacterial plates were incubated at 37 ^{0}C for 24 - 48 h After incubation, the bacterial colonies were observed (Ramakrishnan *et al.*,

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2015) The microbial load in the given sample was calculated using the following formula and it is expressed as Colony Forming Unit (CFU) per gram of the homogenate sample (Bergey's,1984).

Statistics

The data were articulated as arithmetic mean- standard error of mean (S.E.M) and the data were analyzed using the Student's t-test and one-way analysis of variance (ANOVA) using PAST package. Differences N between means were determined and compared by Tukey's test. Significance was also set at 5% level. All treatments (ten fish in each treatment) were assayed in triplicate.

Result

Antiprotease activity

The antiprotease activity of control group in 0^{th} day was recorded as 30.5±1.02 and 35^{th} day was observed as 49.5±0.69 when compared to negative control group value was recorded as 0^{th} day (34.24±0.09) and value on 35^{th} day (15.29±0.09) (Fig.1). The least value of antiprotease activity was observed in the treated group A1 (30.6±0.56) and the highest value of antiprotease activity was observed in the treated group C2 (61.3±0.25). The serum antiprotease activity was slightly increased in control group. Simultaneously, all treated diet groups showed higher level than the control group and the value was elevated maximum in C2 than other treated groups.

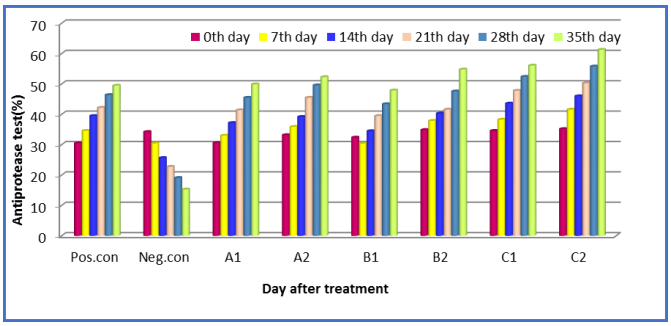

Fig.1: Antiprotease activity of *C. punctatus* fed with different concentrations of *C.auriculata* and *C.dactylon* individually and mixed formulated diet against intraperitoneally injected with 0.2ml of 10^5 CFU/ml of *A. hydrophila*

Total Heterotrophic Bacterial Count

The number of total bacterial colonies varied with the concentrations. The initial observation of bacterial colonies in control group revealed the count as 3.26×10^6 and final observation of bacterial colonies count as 2.7×10^5 when compared to negative control group colony was recorded as 0^{th} day 3.8×10^8 and colony on 35^{th} day as 6.8×10^8. At end of the experimental period, the bacterial count was found to be maximum in B2(4.2×10^7) and minimum colonies count as C2 2.1×10^5. In these, result was indicated the mixture plant powder C2 reduce the bacteria level compared with infected control group.

Table:2: Total heterotrophic bacteria count of *C. punctatus* fed with different concentrations of *C.auriculata* and *C.dactylon* individually and mixed formulated diet against intraperitoneally injected with 0.2ml of 10^5 CFU/ml of *A. hydrophila*

Experimental Groups		Dose (gm)	Days after treatment		
			0	15	30
Positive control (Normal fish)		0	3.2×10^6	2.4×10^6	2.7×10^7
Negative control (*A. hydrophila)*		0	3.8×10^8	5.5×10^8	6.8×10^8
	A1	1	3.7×10^4	3.2×10^5	2.9×10^7
	A2	3	3.6×10^5	4.2×10^5	3.1×10^6
Experimental fish (*A.hydrophila* + plants powder)	B1	1	2.7×10^6	3.2×10^8	2.4×10^6
	B2	3	3.2×10^6	2.3×10^8	4.2×10^7
	C1	1	4.3×10^5	3.6×10^6	3.1×10^7
	C2	3	3.3×10^7	3.8×10^7	2.1×10^5

Discussion

The present study focused on the effect of *C. dactylon* and *C. auricluata* and mixture plants aqueous plant powder alone or in combination administered orally on Antiprotease activity and total hetertropic count in *C. puncatatus*. In the present study,the antiprotease activity level increase in C2 group compared A1, A2, B1, B2 and C1 group and control group the different concentrations of plant powder formulated diet treated fishes. Then total heterotrphic count was reduced colony in experimental group compared with control group. Earlier study, (Rao and Chakrabarti (2004) that the feeding of

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C. catla with *A. aspera* (0.5%) mixed diet for 4 weeks enhanced the level of serum anti-protease level, which might provide resistance against the bacterial pathogens. The *Achyranthes aspera* mixed diet fed with the anti protease activity inhibitor levels were enhanced in *Labeo rohita*, thus the host can defend more strongly against invading pathogens (Rao and Chakrabarti,2004). Mostofa *et al*.,(2008) conducted infection experiment of *Heteropneustes fossilis* with 10^5 and 10^8CFU/fish of *A. hydrophila* and found the highest bacterial load in the kidney, intestine and liver of the experimentally infected fish to be 1.3×10^7 CFU/g, 3.5×10^6 CFU/g and 2.42×10^7 CFU/g and the lowest bacterial load to be 2.1×10^2 CFU/g, 9.0×10^3 CFU/g and 2.0×10^4 CFU/g respectively (Thangamani and Rajendran,2016) study the bacterial strain *Pseudomonas* sp. was predominant followed by the *Aeromonas* sp. in the gut of all the fishes studied. The THB load was found to be high in milk fish. The conclusion of the present study provides the significance of medicinal plant powder of mixture of *C. dactylon* and *C. auriculata* mixed fish diet can activate the disease resistance against *A. hydrophila* in *C. punctatus*.

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