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Feed additives of herbal powder of *Cynodon dactylon* and *Cassia auriculata* treatment of *Aeromonas hydrophila* infection of *Channa punctatus* (Bloch1973)

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Abstract

The present study has been carried out the haematological parameters such as; RBCs, WBCs haemoglobin content and micronucleus test were studied using medicinal plants like *Cynodon dactylon* and *Cassia auriculata* on different concentrations (C. *dactylon;*2g and 3gms *Cassia auriculata* 2gms and 3gms mixture of *C. dactylon* and *C. auriculata* (1:1) 2g, 3g) of formulated diet against 0.3 ml of CFU/ml 10^5 cells of *Aeromonas hydrophila* introduced in the *Channa punctatus.* 2gms of mixture of plants powder formulated diet showed maximum WBCs, RBCs and haemoglobin content than the control and other experimental groups. 2gms of mixture of plants powder formulated diet treated fishes found to be more efficient compared with other experimental and control group. However, the fish showed significant ($p<0.05$) increased when compared with control groups. A high index of micronucleus formation in *C. auriculata* plant group B1(2g) due to the clastogenic effect of *A. hydrophila* were observed while its frequency drastically reduced in mixture plant groups like C1 (2g) and C2 (3g) due to its anti clastogenic effect. Antimutagenic potential of such test plant may be established as potent herbal drug against *A. hydrophila*.

Keywords: *Channa punctatus*, *Cynodon dactylon, Cassia auriculata, Aeromonas hydrophila*

Introduction

Aquaculture is one of the most ecologically important theoretical strategies all over the world. Fish is a worldwide disseminated food commodity and plays a pivotal role in human diet. Since ancient times fish was recognized as being a 'brain food', a reference to its importance in the development of a healthy brain (Patil and Johri, 2016). In 2013, fish accounted for about 17 percent of the global population's intake of animal protein and 6.7 percent of all protein consumed. Moreover, fish provided more than 3.1 billion people with almost 20 % of their average per capita intake of animal protein. Fish is usually high in unsaturated fats and provides health benefits in protection against cardiovascular diseases. It also aids fetal and infant development of the brain and nervous system. With its valuable nutritional properties, it can also play a major role in correcting unbalanced diets and, through substitution, in countering obesity (FAO, 2016).

Fish are palatable and proteinaeceous food for human being. India is now at the threshold of blue revolution and it has made a notable progress in the field of inland fishery. Fishes not only play an important role in the demand of food for humans but they are widely used for various biological experiments (Govind, 2012). Fish production is decreased due to the occurrence of disease caused by different pathogens in aquaculture. Viral diseases have posed significant problems in aquaculture for many years. In commercial aquaculture, antibiotics were used for prevention and control the diseases, and hormones were used for growth performance but the cost of antibiotics and hormones are expensive. Several studies have been carried out to find the new compounds from plant sources at cheap and best to prevent the diseases causing organisms in aquaculture (Sivasankar *et al*.,2015)

Aeromonas hydrophila, the most common bacterial pathogen in fresh water fish, *Aeromonas* species are enteropathogens which possess virulence properties, such as, the ability to produce enterotoxins, cytotoxins, haemolysins and ability to invade epithelial cells. The main virulence factors of *A. hydrophila* species that can be associated with gastro enteritis (Seethalakshmi *et al.,*2008). Vaccines are being developed against *A. hydrophila* but these are not yet commercially available (Govind *et al.,*2012). However, the incidence of drug-resistant bacteria has become a major problem in fish culture (Aoki *et al.,* 1992*).* Natural products like plant extracts might have beneficial effects but cause no problems (Citarasu *et al.,*2002, Sagdic and Ozcan, 2003). Whole plants and plant parts are being used as therapeutic agents to treat the several bacterial diseases. *Cynodon dactylon* (L) Pers. is belongs to the family Poaceae). It is commonly known as 'Arugampul' in Tamilnadu, India. Whole plants of *C. dactylon* is widely used as a traditional healer for purifying the blood, anuria, biliousness, conjuctivitis, diarrhoea, gonorrhoea, itches and stomachache (Muthu Chellaiah, 2006). *Cassia auriculata* commonly called Tanner's Cassia in English and in Tamil as "Avarai". *C. auriculata* (Family: *Cesalpinaceae*) is an evergreen shrub, it is growing in the many parts of India. The plant parts of root, stem, leaves,

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flower and unripe fruit were used for Ayurvedic medicine as a remedy for diabetes, conjunctivitis, joint and muscle pain (rheumatism), opthalmia, jaundice, liver disease, and urinary tract disorders (Joshi,2000). The aim of the present study was to examined in feed additive of herbal powder of *C. dactylon* and *C. auriculata* fed on treatment of *A. hydrophila* infection of *Channa 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 14 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 of *C. punctatus* weighing average body weight of 25-55gm for acclimatization for one week.

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.

Table-1: Preparation and feed composition of experimental diets

Composition of basal diet (g/kg^{-1})	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 powder ($mg.kg^{-1}$)	0	2	3	2	3	2	3

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 multivitamin and mineral tablets were added. Six experimental diets were prepared by adding 2 and 3 grams of plant powders to the semi moist dough and the feed without plant powder was kept as control. (Karpagam and Krishnaveni,2014).

Bacterial strain

Bacterial stain of *Aeromonas hydrophila* was gram negative, facultative anaerobic rod shaped bacteria, it 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 25-55gm for acclimatization for 15 days. Bacterial suspension of *A. hydrophila* ($6x10^5$) induced in healthy fishes of *C. punctatus*. After three day virulent strains of *Aeromonas hydrophila* was produced symptom of disease in experimental fishes. The diseases 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 as 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).

Haematology parameters

The RBC counts were made by Neubauer haemocytometer (Shah and Altindag, 2005). Blood was diluted 1:200 with Hayem's solution (Mishra *et al*., 1977). Erythrocytes were counted in the loaded haemocytometer chamber and total numbers were reported as10^6 mm^{-3} (Wintrobe 1967). Counting was done in the five smaller squares that is, in the 1st, 5th, 13th, 21st and 25th. The RBC's on the lower and right sides of a square were added in the total, while those on the upper and left sides were rejected.

WBC counts were made by Neubauer haemocytometer (Shah and Altindag, 2005). Blood was diluted 1:20 with Turk's diluting fluid and placed in haemocytometer. Four large (1sq mm) corner squares of the haemocytometer were counted under the microscope. The cells touching the boundary lines were not counted. The total number of WBC was calculated in mm^3 x 10^3 (Wintrobe, 1967).

Sahel's Haemometer (Haemoglobinometer) was used. The acid haematin method in which hemoglobin was converted into acid haematin by diluted hydrochloric acid and the brownish yellow colour was matched with the standard in the comparator (Arunkumar *et al., 2*016)

Peripheral blood samples obtained from the caudal vein were smeared on clean, grease free frosted glass slides. Slides were fixed in methanol for 10 mins and left to air-dry at room temperature and finally stained with 6% Giemsa in Sorenson's buffer (pH 6.9) for 20 mins. After dehydration through graded alcohol and clearing in xylene, slides were mounted in DPX (distyrene, plasticizer and xylene). From each slide, 1000 erythrocyte cells were scored under light microscope (LeitzWetzlar Germany, Type 307 - 083.103, oil immersion lens, 100/1.25). The criteria used for the identification of MN were; their smaller size, one-third of the main nucleus, same color, intensity and no attachment with the main nucleus (Singh *et al.,*2014) The NDI was calculated according to Eastmond and Tucker (1989) using the following formula:

$$NDI = \frac{[M1+2(M2)+3(M3)+4(M4)]}{N}$$

where M1–M4 is the number of cells with 1, 2, 3 and 4 nuclei, respectively, and N is the total number of viable cells.

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 (Six fish in each treatment) were assayed in triplicate.

Results

Haematological parameters

In the present investigation, the efficacy of *C. dactylon and C. auriculata* as feed additive supplemented diets against *Aeromonas hydrophila* and on the haematological changes of fish *C. punctatus* was studied. Erythrocytes count has been showed an increasing trend in all experimental groups when compared to control. This increasing trend in statistically significant ($p<0.05$). The highest mean value of C1 (6.23 ± 0.05 $\times 10^6$ cells mm^{-3}) 35^{th} day and the lowest value (2.9 ± 0.13 $\times$ 10^6 cells mm^{-3}) in initial stage group B1. The RBCs count was increased with increasing concentration of plant extract formulated diet in different day of treatment (0,7, 14, 21 28 and 35). The group C1 on the 35^{th} day has showed significant increasing when compared to groups (A1, A2, B1, B2, C2) and control group. The increasing concentration mixture of *C. dactylon and C. auriculata* (2g) in the feed shows the increasing of RBC count in the compared groups.

The leucocytes count (WBC) was varied from both experimental and control fishes. The leucocytes count in the control fishes showed 6.49±0.02 (10^3⁄mm^{-3}) and the plant extract 5 formulated diet treated fishes showed maximum number of WBCs was observed. In mixture plants of *C. dactylon and C. auriculata* (2g) 6.23 ± 0.05 (10^3⁄mm^{-3}) in the initial day (0day) and 12.57±0.01(10^3⁄mm^{-3}) (35 day). According to the results, herbal diet could increase leucocytes count of fish in experimental groups compared to control group.The Hb content was significantly increased ($p<0.05$) in experimental groups compared to control group. The highest mean value of Hb content (13.67 ± 0.02 g dl^{-1}) was observed in control group and (13.11± 0.02 g dl^{-1}) was observed in experimental group C1 on the 35^{th} day and the lowest mean value of (10.33 ± 0.01g dl^{-1}) was observed in initial stage (experimental group A2). The A1, A2, B1, B2 and C2 groups are show the huge increase in the Hb content is different concentration of *C. dactylon and C. auriculata* in the feed of 35th days. According to these results, the total haemoglobin level increased from initial stage (0) to 35 days of feeding in all the experimental diet groups than in the control.

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Table-2: Haematology parameters of *C. punctatus* fed with different concentrations of *C. dactylon* and *C. auriculata* intraperitoneally injected with 0.3 ml of 10^5 CFU / ml of *A. hydrophila*

S.no	Test	Day	Control	Challenged with *Aeromonas hydrophila*					
				A1	A2	B1	B2	C1	C2
1	RBC (10^6/mm^3)	0	3.32±0.04	3.83±0.01	3.21±0.01	2.9±0.13	3.9±0.11	3.62±0.09	3.17±0.01
		7	3.68±0.07	3.88±0.01	3.26±0.01	3.06±0.04	4.08±0.08	3.81±0.07	3.23±0.01
		14	3.92±0.14	3.93±0.01	3.30±0.01	3.15±0.04	4.18±0.08	4.02±0.10	3.27±0.02
		21	4.15±0.02	3.97±0.01	3.39±0.02	3.24±0.06	4.24±0.10	4.36±0.10	3.33±0.01
		28	4.34±0.02	4.05±0.02	3.46±0.02	3.33±0.06	4.15±0.02	4.62±0.14	3.37±0.01
		35	4.65±0.02	4.08±0.01	3.52±0.01	3.43±0.05	4.31±0.05	4.88±0.08	3.41±0.01
2	WBC (10^3/mm^3)	0	6.09±0.02	5.24±0.02	5.52±0.01	4.40±0.04	5.55±0.02	6.23±0.05	5.27±0.01
		7	6.16±0.01	5.46±0.04	4.71±0.04	4.68±0.04	5.75±0.02	6.50±0.05	5.37±0.01
		14	6.20±0.01	5.65±0.02	4.91±0.04	4.83±0.04	5.89±0.02	6.81±0.03	5.48±0.03
		21	6.28±0.02	5.79±0.03	5.12±0.01	5.01±0.03	6.01±0.01	7.05±0.05	5.55±0.02
		28	6.39±0.03	5.86±0.02	5.21±0.04	5.12±0.01	6.15±0.02	7.33±0.04	5.66±0.02
		35	6.49±0.02	5.94±0.03	5.39±0.03	5.25±0.02	6.37±0.01	7.54±0.02	5.73±0.03
3	Hb (g/dl)	0	13.07±0.01	12.03±0.03	10.09±0.03	11.25±0.01	12.11±0.01	12.57±0.01	10.34±0.02
		7	13.17±0.01	12.09±0.01	10.15±0.03	11.33±0.01	12.18±0.02	12.73±0.01	10.38±0.01
		14	13.29±0.01	12.15±0.01	10.19±0.01	11.37±0.01	12.25±0.01	12.87±0.02	10.42±0.02
		21	13.41±0.01	12.20±0.01	10.23±0.01	11.34±0.01	12.21±0.01	12.94±0.02	10.45±0.02
		28	13.52±0.01	12.28±0.01	10.25±0.02	11.43±0.01	12.27±0.01	13.03±0.02	10.47±0.03
		35	13.67±0.02	12.31±0.01	10.33±0.01	11.46±0.01	12.31±0.01	13.11±0.02	10.53±0.01

Micro nucleus test

Channa punctatus the effect of *C. dactylon* and *C. auriculata plants* powder against *A. hydrophila* has been studied. For MNT total 1000 blood cells from control group C. *dactylon (*2g,3g) like A1, A2 and *C. auriculata* (2g and 3g) like B1, B2 and mixture plant extract (2g,3g) C1,C2 were screened. The results are presented in (Table- 2). The mean frequency of MN was observed in control fish value was slightly increases 0^{th} day (0.049±0.002) to 35^{th} day (0.081±0.004). In experimental group was observed the C2 groups was decreased value 0^{th} day (0.123±0.002) to 35^{th} day (0.082±0.001) compared than other experimental groups like (A1,A2,B1,B2 and C1). Table 2 shows the result indicated that the percentages of micronuclei decreased with increase in concentration of plant extracts. The value mentioned above showed a significant decrease when compared to the control ($p<0.05$).

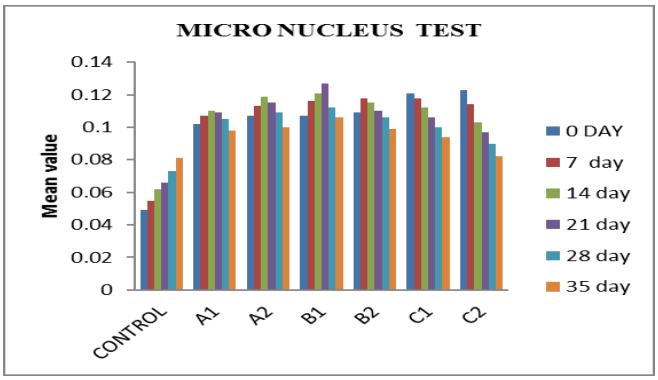
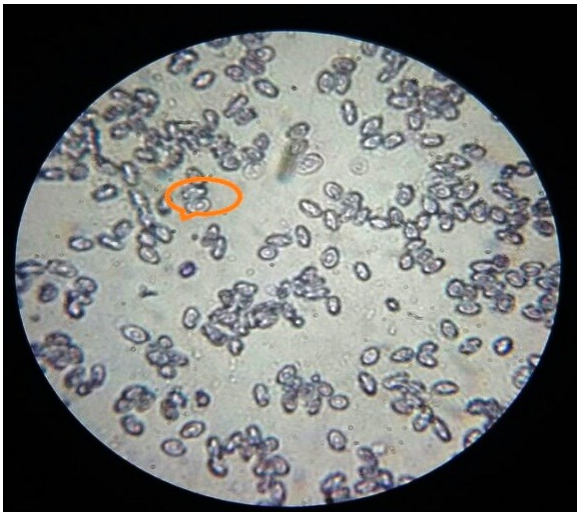


Fig:1 Micro nucleus test of *C. punctatus* fed with different concentrations of *C. dactylon* and *C. auriculata* intraperitoneally injected with 0.3 ml of 10^5 CFU / ml of *A. hydrophila.*



The figure show the arrow mark declared the damage nucleus indicated.

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 the RBC Count, WBC count, heamogloblin and micronucleus test in *Channa puncatatus.* Heamatology parameter increase in C1 group compared A1, A2, B1, B2 and C2 group and control group. In the present study, the different concentrations of plant extract formulated diet treated fishes showed gradually increased the haemoglobin content in different days of treatment. 2gms concentrations of mixture plant powder treated group showed maximum haemoglobin content when compared to control.

Earlier study, Witeska *et al*., (2007) reported that haemoglobin was gradually decreased in *A. hydrophila* infected fishes and it was significantly increased in plant extract formulated diet administered fishes. Thanikachalam *et al*., (2010), showed that the embedding of garlic peel in feed enhances the haematological parameters even at a low level (0.5%) incorporation and makes *Clarias gariepinus*, fingerlings, highly immunopotent and more resistant to infection by *A. hydrophila*. The present investigation RBC count was significantly increased plant extract formulated diets compared with control group. Subeenabegum and Navaraj (2012) reported that serum protein, albumin, globulin, WBC, RBC and haemoglobin content were enhanced in fish fed on herbal diets (*Solanum trilobatum* and *Ocimum sanctum*) against *Aeromonas hydrophila.* In the present study, the WBC count was increased plant extract administered groups. The optimum concentrations (2g) of mixure plant powder formulated diet showed maximum number of WBC were observed. Muthu *et al*., (2015) reported that *Andrographis paniculata* formulated diet treated fishes (*C. carpio*) showed white blood cells when compared to control. Singh *et al.,* (2014) indicated that both are very toxic to C. *punctatus* using herbal formulation of *J. gossypifolia* in a static system. 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*. Concentration of plant extract may also play a major role to enhance the haematology parameters and micronuclus test which afford the dose based clinical knowledge testing. However, the application of mixture *C. dactylon* and *C. auriculata* powder in large-scale field condition requires the detailed study of the route of administration and effective doses for different age group of fish.

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