Influence of plant extract supplemented diet on physiological activities in *Anabas testudineus* (Bloch, 1792)

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Abstract: Herbal feed additives generally improve the productivity of animals through amelioration of feed properties, promote the individual production performance and improve the quality of food derived from those animals. The herbs contain many active components such as polysaccharides, organic acids, alkaloids, glycosides and volatile oils, which can enhance immune functions. Beneficial effects of the dietary intake of two medicinal plant extracts on fish, the climbing perch *Anabas testudineus* were investigated. The diets incorporated with aqueous extracts of Basil (*Ocimum sanctum*) and Betel (*Piper betle*) leaves were used for the study. Food containing different concentrations, 10%, 20%, 30% and 40% of aqueous extracts of these plant leaves was used at a rate of 3% of body weight per day for three weeks. After the administration of plant extract supplemented feed, specific growth rate and feed conversion ratio were found to have increased in all experimental groups. Digestive enzymes such as protease, amylase and lipase activity also altered in fishes fed with aqueous extract supplemented feed and improved the availability of nutrients. Non-specific immune defense is also enhanced by increase in total erythrocyte and total leucocyte count. The aqueous extract of these two herbal plant leaves enhance the growth rate with alteration in digestive enzyme activities and also augment the immune system.

Key words: ***Anabas testudineus,* supplemented diet, *Ocimum sanctum, Piper betle,* protease, amylase, lipase, aqueous extract**

I.INTRODUCTION

Fish is an important part of diet of a large proportion of the world population especially in the developing countries. Fish food products represent the primary source of animal protein for more than a billion peopleworldwide[1]. Fish farmers are attracted towards intensification of culture system for increased production and profit. There are a large number of feed additives available to improve fish growth performance, some of these additives used in feed mill are chemical products especially hormones and antibiotics which may cause unfavorable side effects [2]. The continuous use of antibiotics in fish feed is not promoted due to the potential development of antibiotic resistant human pathogenic bacteria. Disease management in aquaculture needs more environment friendly methods. Plant materials have been used as an alternative for chemotherapeutic agents and also as growth promoters in animal husbandry systems for the reasons that plant materials are cheap, readily available, safe and biodegradable. The therapeutic effect of plants and plant-based additives are reported to be due to the phytochemical substances producing definite physiological actions[3].

Like humans, fish rely on both specific and non-specific mechanisms to protect themselves against invading pathogens. In fish, the primary lines of non-specific defenses are the skin and mucus, when pathogens enter into the body, cellular and humoral non-specific defense is mobilized [4]. Digestive enzyme studies are essential to explain nutrient digestibility in aquatic organisms [5]. The activity of digestive enzymes varies according to the quantity and composition of the diet. Influence of several medicinal plants on physiological activities has been studied by several authors. The use of various herbs such as *Hygrophila spinosa*, *Withaniasomnifera*, *Zingiber officinalis, Solanum trilobatum, Andrographis paniculata, Psoralea corylifolia, Eclipta erecta,Ocimum sacnctum, Picrorhizakurooa, Phyllanthus niruri, Tinospora cordifolia*, have a good influence in the *Penaeus*larviculture due to the effects as feed and growth stimulator, anti-stressor, immunostimulation, and antibacterial characteristics[6,7]. Ethanolic *Aloe vera* extract in the diet can perform as a growth promoter, appetite stimulator and immunostimulant, reduce stress, reduce food losses and protect fish in order to better the growth

of fish[8].This study was conducted to determine effects of dietary administration of different concentration of aqueous leaf extract of *Ocimum sanctum* and *Piper betle* on specific growth rate, digestive enzymes activity and blood cell count.

Since the use of herbs promotes good effects, various commercial herbal additives have been introduced in aquaculture. In contrast to chemical substances, plants or plant-derived substances are natural, non-toxic, biodegradable and biocompatible. The medicinal plants and their products are reported to exert growth promoter, antitumor, antibacterial, antifungal, antiviral, immune-stimulant, anti-inflammatory, antidiabetic, antioxidant and anti-stress effects in animals*in vitro* and *in vivo*[9]. Immunomodulatory effect of *O. sanctum* has been reported for various animal species [10]. Feed containing *O. sanctum* extract at the rate of 200 mg kg^{-1} significantly reduced the mortalities of juvenile greasy grouper (*Epinephelustauvina*) against the *Vibrio harveyi* infection [11]. Logambal*et al*., (2000) [12] studied that dietary intake of *O. sanctum* also enhanced the antibody response and disease resistance against *Aeromonashydrophila* infection in *Oreochromis mossambicus*. *Piper betle* extract has also been found to be effective against the four pathogenic bacteria that have been reported to cause diseases in tilapia and striped catfish including *Aeromonasveronii*, a newly reported pathogen in farmed tilapia [13]. Antibacterial activity of *Piper betle* extract against several pathogens in fishes has been reported [14].

II. MATERIALS AND METHOD

Healthy living species of *Anabas testudineus* weighing about 3-6gm and 5-6cm in length were collected from the Aqua Fish aquarium Kottakkal, Malappuram, Kerala and acclimatized them to laboratory conditions for a period of one week before the experimentation. The fecal matter and other wastes were removed to reduce the ammonia content in water. Fishes were divided into two experimental and control, fed with commercial feed supplemented with plant extract and commercial feed respectively. Fishes were fed with 3% of their body weight twice a day. Experimental group is also divided again into two, one group fed with different concentration of aqueous plant leaves extract of basil and others fed with aqueous plant leaves extract of betel.

2.1. Preparation of plant extract

The fresh leaves of *Piper betle*and *Ocimum sanctum* were collected and shadedried under normal environmental conditions, ground into uniform powder using mixer. The leaves of *P. betle* and *O. sanctum* were powdered separately. The aqueous extract of both leaves were prepared using the Magnetic stirrer at room temperature. The filtrate was collected and the solvent was removed by evaporation. The residues obtained after evaporation was taken in different quantities for the preparation of plant extract supplemented feed.

2.2. Growth performance

The mean average weight and length of fishes of each group were determined at the beginning of the experiment and after 20 days of the experiment and length was measured by normal scale. Based on recording the weight of each fish, Weight Gain (WG), specific growth rate (SGR), feed conversion ratio (FCR) and Food Conversion Efficiency (FCE) were calculated using the standard equations[15].

2.3. Isolation and homogenization of digestive tract

The whole digestive tract was isolated and homogenized at 4ºC using ice cold distilled water (1:5 ration w/v). The contents were centrifuged at 4ºC at 10000 rpm for 20 min and the supernatant was used for the estimation of digestive enzymes.

2.4. Protease activity

Protease activity in gut was determined by method of Cupp-Enyard, 2008[16]. The reaction solutions were prepared by adding 5 ml of 0.65% Casein solution (6.5 mg ml-1casein in 50 mM potassium phosphate buffer) in tubes and equilibrated them on water bath at 37°C for 5 min. Then enzyme solution was added. After that, 5 ml of TCA reagent was added to stop the reaction. Then known volume of enzyme solution were added to each tube and in the blank and incubated for 30 min at 37°C. For tyrosine standard solution, 1 mM tyrosine standard stock solution was added in the tubes at following volumes in ml: 0.05, 0.10, 0.20, 0.40, 0.50 and so on and then distilled water was added to make a final volume of 2 ml. After 30 min of incubation, all samples and blank solution was filtered and final volume of samples used after filtration was 2 ml. Then sodiumcarbonate (500 Mm) was added to the samples, standards

and blank solution, then Folin's Reagent(0.5 Mm) was added and absorbance of the samples were measured by spectrophotometer at 660 nm. Specific activity is expressed in micromole of hydrolyzed tyrosine per minute per milligram of protein (U mg^{-1} protein).

2.5. Amylase activity

Amylase was assayed in 0.2 M citrate/ phosphate buffer solution of pH 7.0, with starch 50g as substrate (Bernfeld, 1955)[17]. Both substrate and enzyme free control was run. The reaction tubes were incubated at 25°C for a period previously established for each tissue, and stopped by the addition of 50g/kg ZnSO4/0.3N of $Ba(OH)_2$. The reaction mixture was centrifuged at 10,000 rpm for 3min, and free glucose was determined in the supernatant at 690 nm. Specific activity was expressed in µmol of reducing sugars per min per mg of protein (U mg^{-1} of protein).

2.6. Lipase activity

Lipase determination was adapted from Albro*et al*., (1985)[18]. Reactions were incubated with 0.4mM P-nitrophenyl myristate in 24 mM ammonium bicarbonate (pH 7.8)with 0.5% Triton X-100.Controlreactions were run without enzyme andwithout substrate. The reaction was stopped by addition of NaOH to a final concentration of 10mM and the optical density was registered at 405nm for 30 min. One unit was defined as micromole of substrate hydrolyzed per min and expressed per milligram protein (U/mg protein).

2.7. Blood cell count

After 20 days of experimentation the fishes were sacrificed for blood and was collected from the caudal peduncle of fishes in 5 ml graduated syringe of all the three groups and the blood was transferred into small vials containing EDTA as anticoagulant for the determination of total RBC count and WBC count. Total erythrocyte count and leucocyte count were calculated by Angelov*et al*., (1999)[19] and Faggio*et al.*, 2013[20].

III. RESULTS

Growth performance parameters at the end of the 20 days of experiment are shown inTable 1. Specific growth rate (SGR), feed conversion ratio (FCR) and feed conversion efficiency was influenced by diet containing various levels of leaf extract of *O.sanctum* and *P.betle*. Highest values were observed in fishes having the diet containing 40g/kg plant leaf extract. Total erythrocyte and leucocyte counts are shown in the Table 2. When compared to the control, fish having plant extract supplemented diet exhibited significant increase in their blood cell counts.

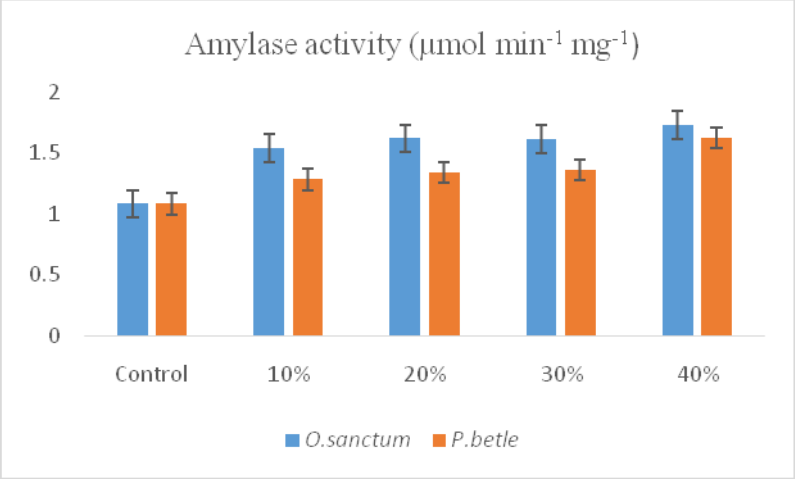

Fig.1: Amylase activity in fishes fed with Plant extracts (*O.sanctum &P.betle*)

Fish showed better digestive enzyme activities following 20 days of plant extract supplemented feed. Amylase activity, protease activity and lipase activity in experimental and control group are provided in figure 1, 2 and 3 respectively.

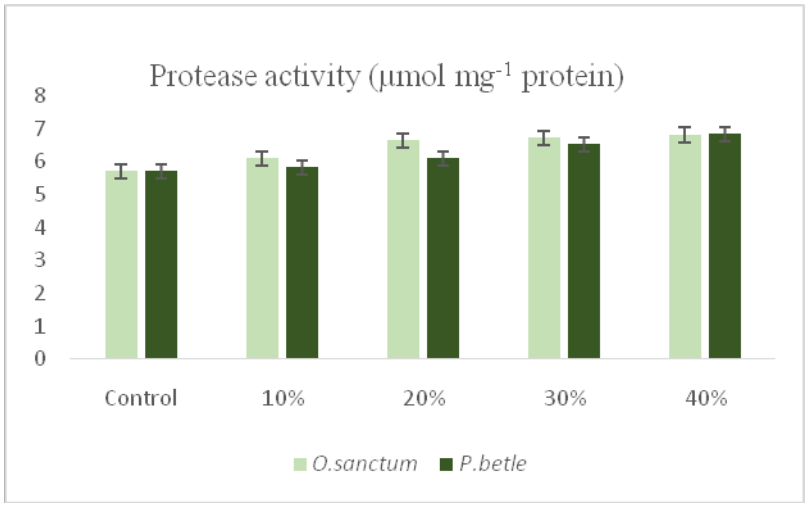

Fig.2: Protease activity in fishes fed with Plant extracts (O.sanctum&P.betle)

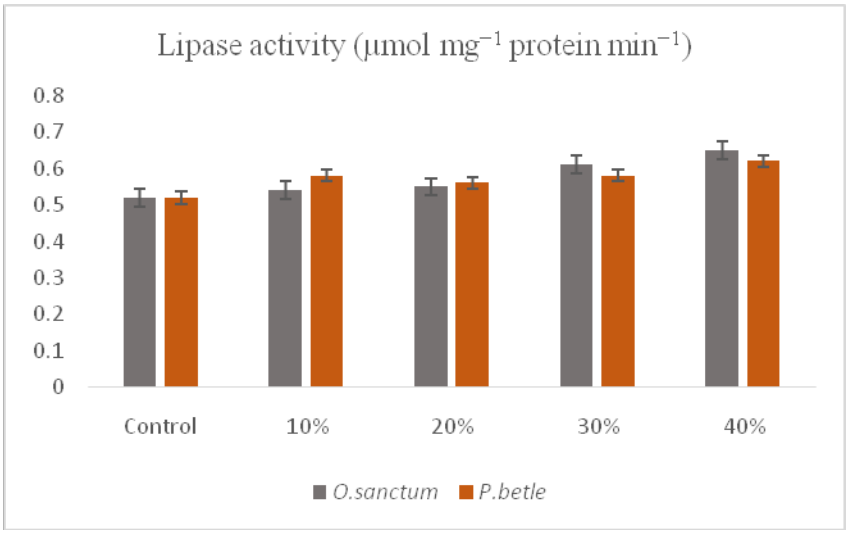

Fig.3: Lipase activity in fishes fed with Plant extracts (O.sanctum&P.betle)

The highest activity of amylase, lipase and protease enzymes were observed in fishes fed with 40g/kg of *O.sanctum* leaf extract when compared to the fishes fed with *P.betle* extract supplemented diet. Influence of *O.sanctum* and *P.betle* leaf extract on lipase activity is almost similar.

Parameters/Treatment	**1ML**	**2ML**	**3ML**	**4ML**	**CONTROL**
Initial weight *O.sanctum*	4.50±0.4	4.70±0.1	4.70±0.2	6.08±0.3	4.20±0.17
P.betle	4.16 ±0.18	4.51±0.16	4.28±0.12	4.39±0.25	
Final weight *O.sanctum*	4.80±0.2	5.02±0.2	5.12±0.1	6.67±0.3	4.43±0.18
P.betle	4.45±0.17	4.85±0.12	4.67±0.15	4.86±0.29	

	6.0±7.4	2.5±5.1	2.23±4.2	8.09±7.3	
SGR *O.sanctum*	0.43±0.11	0.44±0.07	0.57±0.06	0.61±0.16	0.35±0.05
P.betle	0.44±0.07	0.48±0.06	0.58±0.05	0.67±0.12	
FCR *O.sanctum*	0.52±1.9	0.66±1.1	1.31±1.2	1.41±1.5	0.32±1.3
P.betle	0.68±0.7	1.17±0.3	1.34±0.8	1.57±2.3	
FCE *O.sanctum*	1.45±0.3	1.5±1.3	0.77±0.6	0.71±1.2	0.78±0.3
P.betle	1.37±1.2	0.86±0.4	0.74±0.8	0.94±1.2	

Table 1: Growth parameters in fishes fed with the aqueous leaf extract of plants, O.sanctum&P.betle

Table 2: Blood cell parameters in fishes fed with the aqueous leaf extract of *O. sanctum*&*P.betle*

Plant/Parameter	Blood cell count (X106/µl)				
	Control	1ML	2ML	3ML	4ML
RBC *P.betle*(U/mg)	0.905	0.925	0.952	0.998	1.137
WBC	2312.8	2483.6	2584.8	2693.5	2799.5
RBC *O.sanctum*(U/mg)	0.905	0.935	0.976	1.133	1.266
WBC *O.sanctum*(U/mg)	2312.8	2483.6	2586.3	2700	2813.3

IV. DISCUSSION

Some natural herbs and herbal extracts have positive effects on growth indices[21], [22] while others do not have growth stimulation effects [23]. Contradictory results in growth indices are believed to be linked to the type of herbal species and animals. The present study indicated that growth rate, feed conversion ratio and feed conversion efficiency were influenced by concentration of the plant extract in the diet. Similar results were observed by several researchers. Supplementation with 200 mg ofholy basil extract per kg of diet significantly enhanced the growth and immune system function of Nile tilapia through the cytokine gene expression stimulation and protects the fish against *Streptococcus agalactiae* infection [24]. Moringa leaf can partially replace conventional diets without any depression in growth performance of Nile tilapia (*Oreochromis niloticus*)[25]. In rainbow trout fingerlings, body weight gain and growth performance were significantly higher in fish supplemented with *Echinaceapurpurea* than in the control group [26].

Herbal medicine improve the immune system and increase the host's resistance to disease via increasing the number of white blood cells and production of antibodies [27]. Augmentation in total erythrocyte and leucocyte count in anabas fed with *O.sanctum* and *P.betle* leaf extract supplemented feedwere also observed. The high concentration of plant extract formulated feed shown to produce maximum number of WBC cells in tested fish. Similar results were also reported [28], where WBC counts increased with increasing concentrations of *Rheum rebis* extract treated (*Rutilus frisiikutum*) fishes. *Aeromonas hydrophila* infections in catfish juveniles were controlled by spraying *Piper betle* leaf extract on fish feed[29]. The increase in erythrocyte levelsis associated with the oxygen transportation and distribution throughout the fish body, whereas the increase in leukocytes is

linked to improved phagocytosis and internal defense mechanisms against pathogens clearly indicating the improved health status of the fish. These results were also supported by Innocent *et al.,*(2011)[21] who reported that the WBC count was increased with increasing concentration of leaf extract *Plumbago rosea* formulated diet treated with disease induced *Catlacatla.* White blood cells afford protection against infectious agent caused by microbial and chemical factors. Dada (2012)[30] reported that white blood cell counts were significantly higher when herbal growth promoter feed additives are used in fish meal of Nile Tilapia, *Oreochromis niloticus* (L.).

Some of the plant extracts possess immunostimulant and antibacterial activities that can enhance the nonspecific immunity and thereby inhibit bacterial and virus growth [31], [32], [33]. Phagocytic cells play a pivotal role in nonspecific immunity of fishes. Various phagocytic cells such as monocyte, neutrophil, and lymphocyte hold a similar function in engulfing and destroying the pathogen. Herbal plant extract has been reported to function as an immunostimulant in fish and has been shown to increase phagocytosis, for example in golden fish [34], [35], [36], [37]. Besides plant extracts, spirulina [38] supplementation can increase fish phagocytic index, thereby allowing the fish to eliminate the pathogen more effectively. Several plant extracts such as those from O. *sanctum*, *Emblica officinalis*, *Cynodondactylon*, and *Adhatoda vasica* exhibit immunostimulant activity in aquaculture and are able to suppress the bacterial infection in goldfish (*Carassius auratus*) [39].

Supplementation of medicinal herbs has increased the activities of digestive enzymes. High protease activity indicates protein catabolism, while lipase activity is indicative of lipid use and amylase activity suggests carbohydrate (i.e. starch and glycogen) catabolism [40]. The increases in enzyme production can result in improvements in digestibility and availability of nutrients from feedstuffs [41]. Digestive enzymes, protease, lipase and amylase activity were enhanced in anabas fed with both plant extracts and it depends on the concentration of the plant extracts administered. Animals rely on a functional digestive system to efficiently utilize the nutrients present in the food [42], and the morphology of the digestive tract, the physiological conditions of the larvae and the rearing environment all play major roles in determining the digestibility of foods[42].

V.CONCLUSION

Considering the increasing antibiotic resistance of pathogens,it appears that medicinal plants are promising new means of treating viral, bacterial, and parasitic diseases in fishes. This study demonstrates that the dietary administration of aqueous leaf extract of *Ocimum sanctum* and *Piper betel* as feed supplements enhanced growth rate, immunity and digestibility of food in *Anabas testudineus*. The observations affirm the immense scope for use of plant-based feed supplements in fish culture as cheap and environment friendly immuno-stimulants. Further studies are required to identify and isolate bioactive components responsible for the mode of action.

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