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EFFECT OF DIETARY SUPPLEMENTATION OF *CASSIA AURICULATA* LEAF POWDER ON GROWTH AND IMMUNE RESPONSES OF MILKFISH, *CHANOS CHANOS*

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ABSTRACT

A 30 days feeding trial was conducted to evaluate the effects of *Cassia auriculata* leaf powder on growth and immunomological parameters of the juveniles of Milkfish, *Chanos chanos* and disease resistance against *Vibrio anguillarum*. The juvenile fishes with an average weight of 20 ± 3 g were stocked in four treatments each with three replicates. Four practical diets were prepared with graded level of *Cassia auriculata* leaf powder (CAL) at the rate of 0%, 0.5%, 1% and 1.5% and fed to four treatments respectively named as CAL0, CAL0.5, CAL1 and CAL1.5. At the end of the experiment blood samples were collected for immunological parameters. After sampling, the remaining fishes were challenged with 0.1 mL of virulent *V. anguillarum* suspension at a concentration of 10^7 CFU mL^{-1} and mortality was observed for 7 days. Post challenge sampling was performed for the collection of blood samples. The growth parameters such as Weight gain%, SGR%, PER were positively affected by *Cassia auriculata* leaf powder incorporated diet. Higher weight gain %, SGR% and PER were noticed in CAL1.5 group followed by CAL1, CAL0.5 and CAL0. The respiratory burst, lysozyme and phagocytic activities were increased with increasing level of CAL in the diet which indicates better immune response of the fishes fed with CAL. The higher respiratory burst (0.522 ± 0.03), lysozyme (70.07 ± 1.57) and phagocytic (52.91 ± 1.76) activitieswere observed in CAL1.5, CAL1 and CAL1.5 groups respectively during pre-challenge and post challenge. There was significant difference in the survival rate of control (CAL0) and CAL incorporated diet fed groups ($P < 0.05$). Maximum survival was witnessed in CAL1 followed by CAL1.5 and least survival was in CAL0 group. The results indicate that diets supplemented with 1% CAL potentially enhance the immune system and effectively protects the host against *V. anguillarum* infection and thereby improve the survivability of the fish against this dreadful pathogen.

INTRODUCTION

Milkfish (*Chanos chanos* Forsskal, 1775) is an important food fish in Southeast Asia. Aquaculture of milkfish was begun in Indonesia over 4-6 centuries back followed by Philippines and Taiwan also entered in to this venture. Milkfish is a marine inhabitant and cultured predominantly in brackishwater ponds followed by oceanic waters and in hypersaline lagoons (Lin *et al.,* 2003). It is a euryhaline species and can thrive well in wide range of salinities from 0 to 158 ‰, which indicates that it can be grown in freshwater, brackishwater and hypersaline environment (Lin *et al.,* 2001). Since milkfish is hardy and fast-growing, large scale culture in brackishwater and marine farms are carried out in Southeast Asian countries (Lazarus and Nadakumaran, 1986). In India, milkfish culture was initiated since 1980's in brackishwater coastal ponds and pens and as capture based aquaculture. During 2015, the seed production of milkfish was standardized in India and hatchery produced seeds were available for aquaculture in brackishwater pond and sea cages. Hence, aquaculture of milkfish got increased since ever due to availability of hatchery produced seeds. Culture of fishes in confined environment with high stocking density cause endured stress in fishes (Seng and Colorni, 2002) and subjected to many diseases that lead to great loss and decrease in fish production. The lacuna in effective disease control mechanism could be the chief limiting factor for the realization of highly stable fish production (Phillip *et al.,* 2000).

Like other fishes, culture of milk fish with high stocking densities in sea cages cause contagious infections by opportunistic bacterial pathogens particularly, *Vibrio anguillarum* infection which leads to vibriosis or red spot disease (Lee, 1995). Red spot disease can be identified by the existence of ulcers on the abdominal region, hemorrhage of eyes, mouth and inner surface of gills which leads to huge mortality. The prevention of disease outbreaks in aquaculture systems are usually attempted by using antibiotics. An indiscriminate application of antibiotics leads to the incidence of drug resistant bacteria as well as affects the quality of fish flesh. Therefore, application of nutraceuticals especially phytochemicals have been considered as potential alternative to antibiotics and proposed as effective remedy for microbial infection in animal

production systems through effective means of immunomodulation. According to Aoki (1992) immun omodulation has been proposed as a potential method to protect the cultured fishes from infectious pathogens by increasing their innate immune system. Herbal medicines are sources of safe, biodegradable and renewable drugs.

In search of potential immunostimulant, it was found that *Cassia auriculata* Linn, a potential herbal, serves as a remedy factor for several illnesses in human beings in Asia from time immemorial. *Cassia auriculata* is a fast growing branched tall, evergreen shrub with reddish brown branches and bright yellow colour flowers. Dried flowers are constituents of terpenoids, tannin, flavonoids, saponin, cardiac glycosides and steroids and leaf of this plant is rich in triterpene, diterpene alcohols and phytol which are being used for medical treatment (Anandan *et al.*, 2011). Historically aerial parts of the plant was used to treat ulcers, fever, diabetes, conjunctivitis, acute toxicity and also has cardioprotective, astringent, anthelminthic, hypolipidemic, antioxidant, antimicrobial and hepato protective activity (Vedavathy and Rao, 1991; Kumar *et al.*, 2002; Pari and Latha, 2002; Kumar *et al.*, 2003; Siva and Krishnamurthy, 2005; Umadevi *et al.*, 2006; Kumaran and Karunakaran, 2007). The potential antibiotic and radical scavenger activity of *Cassia auriculata* might be the action of two compounds Di-pthalate and 1, 2-Benzenedicarboxylic acid (Maneemegalai and Naveen, 2010; Senthilrani and Renukadevi, 2014). However, it was learnt from the intensive literature survey that no research has been conducted to study the immunomodulatory effects of *cassia auriculata* in aquatic animals. Hence, the present study was carried out to evaluate the immunomodualtory potential of *Cassia auriculata* leaf powder in milkfish juveniles.

MATERIAL AND METHODS

Experimental animals

Milkfish (*Chanos chanos* Forsskal, 1775.) juveniles with an average weight of 15 ± 2.5 g were collected from Kovalam backwaters ,Tamilnadu,India with the help of local fishermen. The fishes were immediately transported in plastic sintex™ tanks of 500 L capacity with sufficient aeration to the wet laboratory at Muttukadu Experimental Unit of ICAR-CIBA, Chennai . They were carefully transferred to circular tanks of 2000 L capacity and maintained at 35 ‰ salinity with 25% water exchange daily. The stock was acclimatized under aerated conditions and was fed with formulated sinking pelleted diet containing 35% crude protein and 6% crude fat for a period of fortnight.

Collection of *Cassia auriculata* leaves

Cassia auriculata leaves were collected from grazing lands of Miller Puram, Tuticorin District of Tamil Nadu and were identified as *C. auriculata* with the help of Mr. Jayakumar, Botanist at Karapettai Nadar Higher Secondary School, Tuticorin. The fresh leaves were washed in freshwater to remove any extraneous materials adhering to the leaves followed by shade drying for five days and finally ground finely using mixer grinder. The fine powder of *Cassia auriculata* leaves (CAL) was stored in clean and dry container for further use.

Experimental design and set-up

A total of 240 numbers of juvenile fishes of *C. chanos* with an average weight 20 ± 3 g were randomly distributed in 4 different treatments namely CAL0, CAL0.5, CAL1 and CAL1.5 with 3 replicates each. The fishes were maintained in 500 L capacity tank with 400 L of water. Four different feeds were prepared with incorporation of 0%, 0.5%, 1% and 1.5% *Cassia auriculata* leaf powder. The aeration was given incessantly to all the tanks throughout the study. Siphoning of uneaten feeds and faecal matter were done on daily basis. Complete water exchange was done on alternate days. The experimental fishes were fed at apparent satiation. The daily ration was offered in two frequencies at 09:00 and 17:00 hrs. During the experiment 12:12 h light and dark photoperiod cycle was maintained. Water quality parameters (temperature, dissolved oxygen, pH, total hardness, total alkalinity, carbon-di-oxide, ammonia, nitrite–nitrogen, and nitrate–nitrogen) were checked every week through standard methods (APHA 1998).

Formulation and preparation of experimental diets

There were 4 practical diets prepared with the crude protein and lipid levels at 35% and 6% respectively with graded levels of *Cassia auriculata* leaf powder (CAL) at the incorporation level of 0%, 0.5%, 1% and 1.5% (Table 1). The experimental diets were prepared using the ingredients which includes soy bean meal, fish meal, acetes meal, wheat gluten, groundnut oil cake, sunflower oil cake, wheat flour, de-oiled rice bran, fish oil, soy lecithin, binder and other additives. All the ingredients were weighed properly as per the requirement and were kept in big plastic containers. Water was added to the ingredients inorder to make the dough and was transferred to an aluminum container, which was placed in an autoclave for cooking/steaming for 20 min. The cooked dough was then removed from the autoclave and kept aside for cooling. When the steamed dough was completely cooled, vitamin C and butylated hydroxytoluene were added so as to prevent their loss during steaming. After incorporation of these elements, the dough was mixed properly and was pressed through the die of a motor driven pelletizer to get uniform sized pellets, which were spread on trays of oven and kept in hot air oven overnight for complete drying at 45 °C. After drying, these pellets were packed in polythene bags, sealed airtight and labeled according to the treatments. Proximate analysis of feeds and feed ingredients were performed as per the prescribed method of AOAC (1995) in the Nutrition Laboratory of ICAR-CIBA.

Blood collection

After completion of the feeding experiment some of the fishes from each treatment were taken for biochemical and immunological analysis. The blood and serum were collected from experimental fish using a 26 gauge needle of 1 mL Tuberculin syringe by inserting it into the caudal peduncle region.

Immunological parameters

Nitroblue tetrazolium (NBT) assay

The respiratory burst activity was estimated by Nitroblue tetrazolium assay which was done by the method of Stasiack and Baumann, 1996. Fifty microliter of blood was placed into the wells of flat bottom microtitre plates and incubated at 37 ºC for 1 h to facilitate adhesion of cells. Then the supernatant

was removed and the loaded wells were washed thrice in phosphate buffer saline (PBS). After washing, 50 µl of 0.2% NBT was added and was incubated for 1 h. The cell was then fixed with 100% methanol for 2-3 min and was again washed thrice with 30% methanol. The plates were then air dried. Sixty microlitres of 2N potassium hydroxide and 70 microlitres dimethyl sulphoxide were added into each well to dissolve the formazon blue precipitate formed. The OD of the turquoise blue coloured solution was then read in ELISA reader at 620 nm.

Serum lysozyme activity

In the 96 well U bottom microtitre plate 150 µL of *Micrococcus luteus* suspension in phosphate buffer (A_{450} = 0.5-0.7) was taken and 15 µL of serum samples were added. The absorbance was taken immediately at 450 nm in ELISA reader and the plate was incubated at 25 °C for 1 h and final absorbance was taken. This absorbance was compared with standard lysozyme of a known activity following the same procedure as above. The activity was expressed as U min^{-1} mg $protein^{-1}$(Parry *et al*., 1990).

Phagocytic activity

Phagocytic cells were detected by using *Staphylococcus aureus* (Aquatic Animal Health and Environment Division, ICAR-CIBA, Chennai) as described by Anderson and Siwicki, 1995. A sample of 0.1 mL of serum was taken in wells of microtiter plate and 0.1 mL of *S. aureus* ($1x10^7$ cells) suspended in phosphate buffer saline (pH 7.2), was added and shaken sufficiently. This bacterial serum mixture was incubated at room temperature for 20 min. From this mixture, 5 µL was taken on a clean glass slide and a smear was prepared. The smear was air dried and fixed with 95 % of ethanol for 5 min and air dried once again. The smear was stained with Diû-Quick stain for 10 min. A total of two hundred haemocytes from each smear were observed under the light microscope and the number of phagocytizing cells and bacteria engulfed by the phagocytes were counted. Phagocytic activity was expressed as the number of phagocytizing cells divided by the total number of phagocytes counted.

Challenge Study

After 30 days of feeding experiment, fishes from each experimental group were challenged with virulent *Vibrio anguillarum* obtained from Aquatic Animal Health and Environment Division, CIBA. First the pathogenic isolates of *V. anguillarum* were grown on nutrient broth for 24 h at 30 °C in a BOD incubator. The *V. anguillarum* cells were harvested by centrifuging the culture broth at 4000 rpm for 10 minutes at 4 °C. The cells were then washed thrice in sterile PBS (pH 7.2) and finally maintained in PBS at a concentration of 10^7 CFU mL^{-1}. The fishes in each experimental group were injected 0.2 mL of bacterial suspension. Mortality was observed for 7 days.

Statistical analysis

The data were statistically analyzed by statistical package SPSS version 16 in which data were subjected to one way ANOVA and Duncan's multiple range tests was used to determine the significant differences between the means. Comparisons were made at 5 % probability level.

RESULTS

Growth parameters

The growth parameters include weight gain percentage, SGR, FCR and PER are shown in Fig 1 & 2. The weight gain percentage was significantly different ($P<0.05$) among the treatment groups but no significance exists between CAL1

Table 1: Feed formula and proximate composition of feeds

Ingredients (g/Kg of feed)	CAL0	CAL0.5	CAL1	CAL1.5
Soybean meal[1]	310.00	310.00	310.00	310.00
Fish meal[2]	100.00	100.00	100.00	100.00
Ground nut oil cake[3]	155.00	155.00	155.00	155.00
Sunflower oil cake[4]	97.50	97.50	97.50	97.50
Wheat flour[5]	149.50	149.50	149.50	149.50
DORB[6]	90.00	85.00	80.00	75.00
Fish oil [7]	20.00	20.00	20.00	20.00
Sunflower oil[8]	45.00	45.00	45.00	45.00
Choline chloride[9]	2.50	2.50	2.50	2.50
Vitamin C[10]	0.50	0.50	0.50	0.50
Carboxy Methyl Cellulose[11]	10.00	10.00	10.00	10.00
Vitamin and mineral[12]	20.00	20.00	20.00	20.00
Cassia auriculata leaf powder[13]	0.00	05.00	10.00	15.00
Proximate Composition (as is basis)				
Crude Protein (%)	35.43 ± 1.23	35.18± 0.67	35.24 ±1.65	35.09 ± 0.43
Crude Fat (%)	8.10 ±0.27	7.74±0.22	7.90 ±0.45	7.61±0.27
Crude Fibre (%)	4.23 ±0.21	4.40 ±0.19	4.35 ±0.15	4.53 ±0.18
Total ash (%)	8.95 ±0.53	9.06 ±0.21	9.0 ±0.45	9.11 ±0.17
Moisture (%)	10.35 ±0.43	10.64±0.51	10.23 ±0.65	10.78±0.40
NFE (%)	32.85 ±1.23	32.98 ±0.73	32.96 ±1.10	32.88 ±0.66
DE (kcal g^{-1})	3.450 ±0.12	3.423±0.16	3.419 ±0.21	3.403±0.13

NFE-Nitrogen Free Extract; DE-Digestible Energy; [1]Growel Feeds Pvt Ltd., Gudivada, Andhra Pradesh ; [2&7]Raj Fishmeal and Oil Co., Malpe, Mangalore; [3-6,8]From the local market, Chennai [9,11]Hi-Media, Mumbai - [10]Stay – C from DSM Nutritional Technologies, Mumbai; [12]Agrimin from Vibrac Healthcare India, Pvt. Ltd., Mumbai;[13]Collected at Miller puram, Tuticorin, Tamil Nadu

and CAL1.5 and maximum weight gain percentage was recorded in CAL1.5 group. The SGR of the different treatments differ significantly ($P < 0.05$), but there was no significant difference present in *C. auriculata* fed treatments. The lowest SGR value was noticed in CAL0 group and the highest SGR was witnessed in CAL1.5 group. Similar trend was noticed for FCR and PER. The best FCR was recorded in CAL1.5 group. The maximum PER value was recorded in CAL1.5 group and the minimum PER value was found in control (CAL0) group.

Respiratory burst, lysozyme and phagocytic activity

The respiratory burst, lysozyme and phagocytic activity of different treatments were significantly different ($P < 0.05$) among themselves in both pre and post-challenge (Table 2). The maximum and minimum value of respiratory burst, lysozyme and phagocytic activities were observed in CAL1.5 and CAL0 group respectively, in both pre and post-challenge. While comparing the pre-challenge group with the post-challenge group, the values of respiratory burst, lysozyme activity and phagocytic activity were in increasing trend in post-challenge condition. There was significant difference ($P < 0.05$) noticed in respiratory burst and lysozyme activities among pre and post challenge samples.

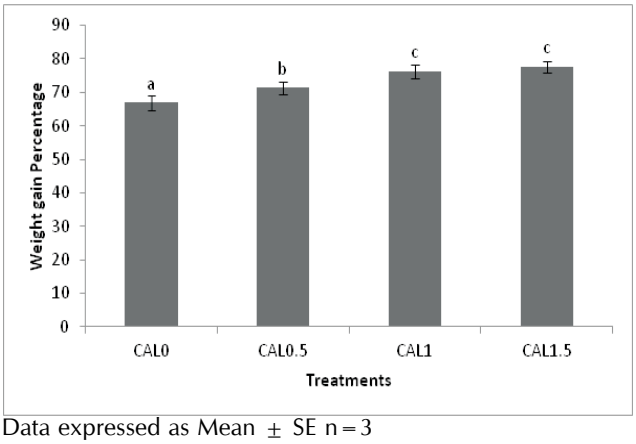
Disease resistance

The mortality was observed up to 7 days after post-challenge. The percentage of survival (%) of *C. chanos* after challenging with *V. anguillarum* in different treatment groups was represented in Fig. 3. There was significant difference between the survival rate of control (CAL0) and immunostimulant incorporated diet fed groups ($p < 0.05$). But there was no significant statistical difference observed between the CAL1 and CAL1.5 groups. The least survival was recorded in control (CAL0) group whereas highest survival was noticed in CAL1 group.

DISCUSSION

In the present study Milk fish growth was positively influenced by *Cassia auriculata* leaf powder (CAL) incorporated diet, as the increase in the level of CAL in the diet showed better growth performance in terms of weight gain percentage (%), specific growth rate (%) and protein efficiency ratio. This was in agreement with the results of Priyadarshini *et al*. (2012) who fed the common carp with polyherbal immunomodulator which improved the weight gain and specific growth rate. Similarly, Rajakumari and Radhika (2015) found better growth rate in Nile Tilapia, *Oreochromis niloticus* fed with *S. cumini* seed powder and Prabu *et al*. (2016) in *Pangasianodon hypophthalmus* fed with fucoidan rich sea weed extract as immunostimulant showed superior growth than the control groups. In poultry also it is reported that use of herbal as feed additive in broiler rations substantially improves the feed utilization and growth performance (Srivastava *et al*. 2013). The better growth attributed may be due to the better utilization of feed through improved digestion, nutrient absorption and resultant carcass accretion and weight gain in the milkfish juveniles fed with CAL incorporated diet.

Lysozyme being a cationic enzyme acts on α-1,4 glycosidic bond between N-acetymuramic acid and N-acetylglucosamine in peptidoglycans of bacterial cell walls that enables lysozymes to kill Gram-positive and in concurrence with complement system to execute Gram-negative pathogenic bacteria also (Alexander and Ingram, 1992; Saurabh and Sahoo, 2008). Lysozyme activity is revealed as a first line innate immune system in fish that results in the disease prevention against the invasion of microbial pathogens. Lysozyme activity is enhanced by the administration of immunostimulants (Engstad *et al.,* 1992). The present result was in accord with the findings of Chakrabarti and Srivastava (2012) which revealed that the dietary supplementation of *Achyranthes aspera* seeds increase the serum lysozyme activity in larvae of *L. rohita* after challenge with *A. hydrophila*. Correspondingly, dietary sodium alginate increases the lysozyme activity in *E. fuscoguttatus* during post



Data expressed as Mean ± SE n = 3
Mean values in the bars with different superscript differ significantly ($P < 0.05$).

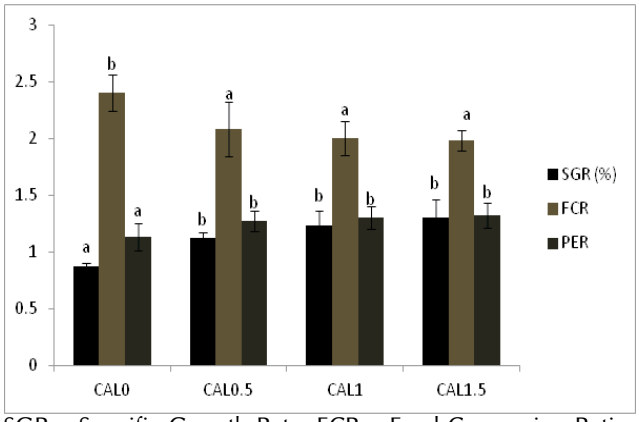
Figure 1: The weight gain percentage of different treatment groups

$$\text{Weight gain}(\%) = \frac{\text{Final weight - Initial weight}}{\text{Initial weight}}$$

Table 2: Selected immunological parameters of *C. chanos* before and after artificial infection with *Vibrio* anguillarum

Treatment	NBT		Lysozyme activity (U min^{-1} mg $protein^{-1}$)		Phagocytic activity (%)	
	Pre-challenge	Post-challenge	Pre-challenge	Post-challenge	Pre-challenge	Post-challenge
CAL0	$0.265^{aA} \pm 0.01$	$0.295^{aB} \pm 0.01$	$51.48^{a} \pm 0.45$	$52.35^{a} \pm 4.04$	$30.47^{a} \pm 1.41$	$27.44^{a} \pm 2.48$
CAL0.5	$0.331^{aA} \pm 0.01$	$0.408^{bB} \pm 0.01$	$64.43^{b} \pm 1.14$	$69.47^{b} \pm 2.63$	$38.91^{bA} \pm 1.33$	$44.37^{bB} \pm 1.08$
CAL1	$0.469^{bA} \pm 0.02$	$0.577^{cB} \pm 0.01$	$70.07^{bA} \pm 1.57$	$85.63^{cB} \pm 3.04$	$49.03^{c} \pm 2.09$	$55.05^{c} \pm 2.70$
CAL1.5	$0.522^{b} \pm 0.03$	$0.602^{c} \pm 0.02$	$68.41^{bA} \pm 4.31$	$93.80^{cB} \pm 2.22$	$52.91^{c} \pm 1.76$	$57.41^{c} \pm 1.37$
P Value	0.001	0.001	0.003	0.001	0.001	0.001

Mean values in the same column with different superscript (a,b,c) differ significantly ($P < 0.05$).; Data expressed as Mean ± SE n = 3; The presence of uppercase alphabets (A,B) in the consecutive rows of same column (pre challenge and post challenge) indicates the significant difference ($P < 0.05$) in pre-challenge and post challenge condition of same parameter



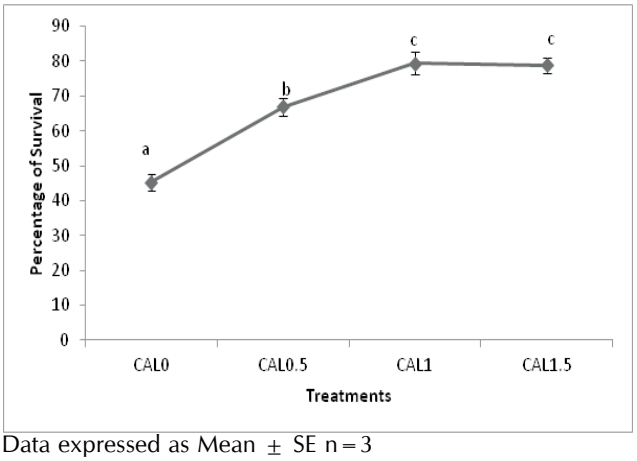
SGR – Specific Growth Rate, FCR – Feed Conversion Ratio, PER – Protein Efficiency Ratio.
Data expressed as Mean ± SE n = 3; Mean values in the same type of bars with different superscript differ significantly ($P < 0.05$).

Figure 2: Growth parameters of different treatment groups

$$\text{Specific Growth Rate (SGR) \%} = \frac{\text{Loge final weight - Loge initial weight}}{\text{Number of days}} \times 100$$

$$\text{Feed Conversion Ratio (FCR)} = \frac{\text{Feed consumption (dry weight)}}{\text{Body weight gain (wet weight)}} \times 100$$

$$\text{Protein Efficiency Ratio (PER)} = \frac{\text{Net weight gain (wet weight)}}{\text{Protein fed (dry weight)}}$$



Data expressed as Mean ± SE n = 3
Mean values in the line with different superscript differ significantly ($P < 0.05$).

Figure 3 : The percentage of survival of different treatment groups challenged with *V. anguillarum*

challenge condition (Chiu *et al.,* 2008).

Phagocytosis is the crucial mechanism of fish defense system by which a cell engulfs diverse particulate targets and some phagocytes particularly macrophages, dendritic cells and neutrophils are provided with a copious repertoire of molecules that are capable of demolishing invading particles and apoptotic bodies besides processing and introducing them to the specific cells which will promote immunoglobulin production to offer immunoprotection (Neumann *et al.,* 2000; Esteban *et al.,* 2015). According to Mathias *et al.* (2009) cytokines are able to release phagocytic cells at the site of inflammation by injured tissue to promote chemotaxis and phagocyte mobilization. In our study, phagocytic activity was higher in CAL fed groups than the control (CAL0) group in pre as well as post challenge condition. This was in harmony with the results of Nya and Austin (2010) who found that application of bacterial lipopolysaccharide (LPS) as an immunostimulant for the control of *Aeromonas hydrophila* infections in rainbow trout, *O. mykiss* through improved non-specific immune response particularly phagocytic activity and phagocytic index. Similar results were also quoted by Prabu *et al.,* (2016) who reported that fucoidan rich extract enhance the immunity and phagocytic activity in *P. hypophthalmus*.

Fish leukocytes increase their oxygen consumption at the time of phagocytosis through NADPH oxidase and generate various reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion radical (O_2^-), singlet oxygen ($^1O_2$) and the hydroxyl radical (OH^-), known as the respiratory burst. These ROS are highly toxic and thus develop the source of a potent antibacterial system in the host (Klebanoff, 1999). Hence, phagocytic cells play an important role in limiting the spreading of infectious agents and destruction of phagocytosed pathogens through the ROS and nitrogenous intermediates (Neumann *et al.,* 2000). Therefore, elevated respiratory burst activity could be positively associated with the augmented bacterial pathogen killing activity of fish phagocytic cells (Sharp and Secombes, 1993). The present result was in agreement with the results of Sivagnanavelmurugan *et al.* (2014) who revealed that the respiratory burst activity of *P. monodon* fed with dietary fucoidan increases after challenge with *V. parahaemolyticus*. Similarly, Prabu *et al.* (2016) found that fucoidan rich extract in the diets of *P. hypophthalmus* augment the pathogen killing activity through the elevated level of respiratory burst activity. In the present study, the experimental fishes were challenged with *V. anguillarum* showed mortality in all the treatments and control group had more mortality than the *C. auriculata* leaf powder incorporated diet fed experimental groups. Maximum survival was noticed in CAL1 group (1% *Cassia auriculata* leaf powder) which was due to the presence of better phagocytic activity, lysozyme activity, respiratory burst activity, SOD, catalase and other stress mitigating enzymes and also better innate immunity.

The results obtained in our study reveals that there were significant differences between most of the studied immune parameters of *Chanos chanos* fed with *Cassia auriculata* leaf powder and their survival was better against *V. anguililarum* infection. Higher phagocytic and lysozyme activities of CAL1 group indicates that this dose was adequate to improve the function of macrophages and cytokine synthesis and in turn control the pathogenicity of *V. anguillarum* and red spot disease owing to the presence of several bioactive compounds in the experimental feed containing 1% *Cassia auriculata* leaf powder. Further research may be warranted to find out the active component which was responsible for immunomoulatory response in milkfish.

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