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Dietary Supplementation of *Aliumcepa*(L.) and its Immunostimulatory Effect on *Poeciliasphenops*

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INTRODUCTION

Aquaculture is a fastest growing food production industry, supported by government and private sectors contributing to its immense growth (Gold burg and Naylor, 2005). It has been estimated that the global fish production through capture fisheries would peak at 100 million tonnes annually. However, the demand for aquaculture products by the year 2025, would be projected to a total of 162 million tonnes (Mandal, 2008; FAO statistics, 2014). In order to meet the increasing demands, fish are grown intensively in a polyculture aquaculture system. Intensive aquaculture involves farming where animals are kept en masse in a restrained space. Under such conditions disease outbreaks are predominant regardless of the hygiene measures practiced. Fish diseases caused by bacteria remain a dominant cause of mortalities and lead to significant financial loss to the industry. *Aeromonas hydrophila*sustains itself as an important bacterial pathogen and cause repeated disease outbreaks in a variety of fish species especially carps, leading to symptoms like ulceration, exophthalmia and abdominal distension (Wang *et al.,* 2013).

In addition the biofilms (complex bacterial mass embedded in slimy matrix) formed by *A. hydrophila* in fish cultivation ponds and reservoirs are known to cause persistent infection to the cultured fish.Most *Aeromonas* spp are opportunistic pathogens and cause infection to fish by entering through wound, while some Aeromonads are capable of infecting healthy fishes even at low bacterial counts (Reith *et al.,* 2008). Outbreaks by *Aeromonas* often occur when fish immune system is compromised due to factors such as overcrowding, poor water quality, organic pollution, and hypoxia in conjunction with other diseases (Zhang *et al.,* 2000). Although, vaccines prove to be a valuable source for preventing the disease outbreaks, several considerable facts in terms of efficacy and cost makes its application more complex. In such cases, antibiotics are used to treat bacterial infection. Administering antibiotics has its demerits such that it enters the environment via water columns leading to unaccounted increase in the antibiotic resistant pathogens in the environment. However,numerous registered antibiotics such as (fluoro) quinolones, tetracyclines, penicillins, amphenicols and macrolides are administered worldwide to prevent and treat bacterial diesase outbreaks in aquatic farms (Shao, 2001). Several studies have reviewed the significance of the global use

antibiotics in aquaculture on the human and environmental welfare (Haya *et al.,* 2000; Angulo *et al.,* 2004; Darsini*et al.,* 2014).

Disproportionate use of antibiotics in aquaculture industries leads to prevalence of residual antibiotics in commercialized fish and shellfish products (Cabello, 2006). Research has evidently shown that the consumption of food products derived from antibiotic treated fish can lead to the development of antibiotic resistance in the intestinal microbiota. Also, the indiscriminate use of antibiotics in the aquaculture sector generates resistance among commensals in the water columns thereby posing a potential horizontal transfer of antibiotic resistance genes among microbes leading to frequent and highly infectious human infections via the food chain (Serrano, 2005). On the other hand, the use of hormones, vitamins and other chemicals during fish culture for various purposes poses significant health risk upon human consumption (Fauci, 1993). One of the promising alternative method for controlling fish disease is by enhancing the fish defence mechanism. Innate immunity play a significant role at all stages of fish infection as they highly depend on the non-specific immunity, than mammals (Ashley, 2006). Recent reports have emphasized on a different perspective of managing the disease outbreaks by using herbal bio-medicinal products with well-known beneficial properties such as growth enhancement, appetizer, and immunostimulant activities (Citarasu*et al.,* 2010; Vaseeharan and Thaya, 2014).

Onion (*Allium cepa*) contains many organic di-sulfides (Metwally, 2009). *Allium cepa*also has potential anti-inflammatory, anti-cholesterol, antioxidant and anti-cancer properties. Garlic and onion has several beneficial actions including antihypertensive, antithrombotic, antibiotic and anti-carcinogenic effects (Sivam, 2001). It also contains small quantities of sugar, fats, vitamin, C and B complex, high content of Mg, K and Cu. Simultaneously, it have been considered a digestive material and used to improve the appetite. Growth improvements in aquaculture have led to the use of synthetic antibiotics. The demand for animal protein for human consumption is currently on the rise and is largely supplied from terrestrial farm animals. Today's world population is estimated to be about 6.5million, but is predicated to reach 9 billion by 2050 (United Nations, 2006). To feed this mass of people, society must develop creative methods to provide a sufficient amount of fish seed, guard against disease outbreak and wastage (Kleijnen*et al.,* 2010). In view of the above mentioned facts, the present study was attempted to utilize and evaluate the bulb of *Allium cepa*, for its immunostimulant properties when given as feed supplement to *Poeciliasphenops*(Black Molly), an ornamental fish.

REVIEW OF LITERATURE

Immunostimulants

Immunostimulants are defined as, "a chemical, drug, stressor or action that enhances the innate or non-specific immune response by interacting directly with cells of the system activating them". Generally, "immunostimulants are promising dietary supplements to potentially aid in disease control of several organisms including marine fish and increase disease resistance by causing up regulation of host defense mechanisms against opportunistic pathogen in the environment" (Galindo-Villegas and Hosokawa, 2004). Immunostimulants stimulate the acquired immune response along with innate, humoral and cellular immune mechanism thereby aiding in enhancing the resistance of the host toward various stressors. These compounds are extensively attributed to their safe, eco-friendly characteristics in addition to their broader efficacy (Sakai, 1999). It is essential to note that immunostimulants are widely suggested as a prophylactic measure before disease outbreaks in aquaculture sector to significantly reduce disease related loss (Anderson, 1992; FAO 2012)

Types of immunostimulants

The immune elicitation of fish has been effectively enhanced by many immunostimulant compounds, of which most of these compounds were classified either according to their vital action on the immune system (i.e., inflammatory agent and/or immune cell stimulant) or the source, namely bacterial derivatives, plant derivative, animal derivatives, nutritional component, hormones and synthetic chemicals (Anderson, 1992; Galeotti, 1988).

Synthetic compounds

Levamisole is one of the well-known antihelmintic drug used for the treatment of nematode infection in humans and animals. Random observations revealed that use of levamisole enhanced the resistance against different infections upon scheduled treatment. Consequently, rain brow trout showed resistance to *Y. ruckeri* when exposed levamisole at varying concentrations ranging from 5 to 25 µg/ml for 2 h (Ispir, 2009). Common carp fingerlings were observed with enhanced resistance to intraperitoneally injected *A. hydrophila* after an experimental dietary levamisole feeding of 250 mg/kg of body weight. Further, the effectiveness of the levamisole was observed to enhance the non-specific immunity of carp fingerlings at range from 100 to 500 mg/kg (Maqsood *et al.,* 2009).
In another study, a peptide related to lactoyl tetrapeptide (FK-156) namely FK-565 [heptanoyl-y-D-glutamyl-(L)-meso-diaminopimelyl-(D)-alanine], was observed to increase the resistance of rainbow trout against *A. salmonicida* (Kitao and Yoshida, 1986).

Bacterial and viral derivatives

An array of compounds derived from bacteria have been studied for the immunostimulant properties. N-acetyl-muramyl-L-alanyl-D-isoglutamine, commonly known as muramyl dipeptide (MDP) derived from Mycobacterium was reported to enhance the respiratory burst, phagocytosis, extravasation of kidney leucocytes and the overall resistance of the fish against *A. salmonicida* challenge (Kodama *et al.,* 1993). Gram positive bacteria are known to consist of lipopolysaccharides (LPS) as an integral part of their cell wall. LPS was efficient in stimulating the innate immune response of rainbow and also preventing the disease caused by *A. hydrophila* (Nya and Austin, 2010).

A report by Selvaraj *et al.,* (2005) elucidated that LPS administered via bathing and injection methods effectively offered protection against *A. hydrophila*infection in carps by stimulating the nonspecific cellular and secondary immune response. Moreover, an increase in lysozyme, myeloperoxidase, total globulin level and respiratory burst activities were observed in *L. rohita* after intraperitoneal administration of LPS (Nayak *et al.,* 2008). Another well-established immunostimulant is β-Glucans, a polysaccharide of D-glucose monomers linked by β-glycosidic bonds present in the cell wall of *Saccharomyces cerevisiae* (bakers yeast) and bacteria. Higher levels of phagocytosis and oxidative radical production was observed in rainbow trout after feeding with β -glucans for four weeks (Volpatti*et al.,* 1998). Furthermore, highest antibody titre against *A. hydrophila*following injection with β-glucans (100–1000 µg /fish) was observed in rainbow trout (Selvaraj *et al.,* 2005). Robertson *et al.,* (1990) observed an increased resistance in Atlantic salmon against three pathogens namely *V. anguillarum, V. salmonicida*and *Y. ruckeri* after intraperitoneal injection of β-glucans derived from cell walls of *Saccharomyces cerevisiae*.

Dietary supplementation with different concentrations of β -1,3 glucan in large yellow croaker, *Pseudosciaenacrocea,* significantly increased the serum lysozyme activity which depended on dietary glucan concentration whereas, in fish fed with lower doses β -1,3 glucan immune activity significantly decreased (Ai *et al.,* 2006). Freund's complete adjuvant (FCA) is a mineral oil adjuvant consisting of killed *Mycobacterium butyricum*. FCA-injected rainbow trout showed resistance against furunculosis, vibriosis and ERM (Adams *et al.,* 1988). Similarly, FCA was observed to enhance the phagocytic activity, respiratory burst and natural killer cell activity of leucocytes in rainbow trout challenged with *V. anguillarum* (Kajita*et al.,* 1992). Sakai *et al.,* (1995a) reported an evidence of herd immunity against *Streptococcus* infection in rainbow trout immunized with *V. anguillarum* vaccine. Also, the inactivated whole cell vaccine prepared from *V. anguillarum* is the most efficient vaccine for salmonid fish when administered by oral dosing, immersion and injection methods of vaccination (Sakai, 1999). Bivalent formalin inactivated whole cells preparation of *Lactococcus garvieae* and *A. hydrophila* enhanced the relative percentage survival of rainbow

trout up to 95 % against *A. hydrophila* (Bastardo *et al.,* 2012). In rohu, attenuated vaccines of *A. hydrophila*conferred immune protection against the pathogen when administered intraperitoneally (Swain *et al.,* 2010). Similar results of immune enhancement was observed in rohu administered with formalin inactivated vaccine of *A. hydrophila* along with adjuvantfor two weeks (Dash *et al.,* 2011).

Nowadays comprehensive range of vaccines for bacterial infections in fish are available and consist of classical winter ulcer (*Moritellaviscosa*), yersiniosis (*Yersiniaruckeri*), edwardsiellosis (*Edwardsiellaictaluri*), vibriosis (*Listonellaanguillarum, Vibrio ordalii*), cold-water vibriosis (*Vibriosalmonicida*), furunculosis (*Aeromonas salmonicida* subsp. *salmonicida*), and streptococcosis/lactococcosis (*Streptococcusiniae*, *Lactococcusgarviae*). Furthermore, experimental vaccines are used under controlled conditions for infections caused by *Photobacteriumdamsela* subsp. *damsela* and*Vibrioharveyi* in barramundi, bacterial kidney disease in salmonids, and *Flexibactermaritimus* infection in (now: *Tenacibaculummaritimum*) in turbot (Gudding and Evesen, 2005). To date fewer viral vaccines are commercially available and no parasite vaccines exist. Although, substantial efficacy data are available and advanced technology has been implemented in the production of novel fish vaccines several hurdles have to be overcome before commercialization of such vaccines (Sommerset*et al.,* 2014).

Polysaccharides

Chitin forms the principal component of crustaceans, insect exoskeletons, and some fungal cell wall (Sakai, 1999). Sakai *et al.,* (1992) reported that chitin stimulated the phagocytic activities and increased resistance to *V. anguillarum*infection in rainbow trout upon intra peritoneal injection. In addition, chitin enhanced the protection against *Photobacterium damselae*subsp. *piscicida* in yellow tail croaker with prolonged resistance for 45 days (Kawakami *et al.,* 1998). On the other hand, the de-N-acetylated derivative of chitin, namely chitosan, when administered via immersion method, showed increased protection against *A. salmonicida*infection in Brook trout (*Salvelinus fontinalis*) (Anderson and Siwicki, 1994). Similarly, rainbow trout treated with chitosan by injection or immersion demonstrated increases in immunological parameters in the blood such as myeloperoxidase activity, respiratory burst activity and antibody titre (Anderson *et al.,* 1995). Harikrishnan*et al.,* (2012) reported enhanced innate immune response in *Epinephelusbruneus* against *Philasteridesdicentrarchi* when intraperitoneally immunized with chitin and chitosan solution.

Nutritional factors

Blazer and Wolke, (1984) demonstrated that vitamin E deficiency in trout resulted in reduced protection against *Y. ruckeri*. Dietary administration of vitamin C in teleost, significantly increased the

biological activities of the blood leucocytes. Higher doses of dietary vitamin C in dietary elicited protection against challenge with *A. hydrophila*(Tewary and Patra, 2008). Moreover, in yellow croaker (*Pseudosciaenacrocea*), the activities of alternative complement pathway, serum lysozyme, and non-specific immune response of head kidney significantly increased and dose dependant when fed with vitamin C. Challenge experiments with *V. harveyi*elucidated that fish fed with vitamin C had significantly lower cumulative mortality as compared to the control (Ai *et al.,* 2007).

Hormones and cytokines

Research on growth hormones elucidated that exogenous growth hormone stimulated the enhanced production of superoxide anion in leucocytes of rainbow trout (Sakai *et al.,* 1995b; Sakai *et al.,* 1995c). Addition of homogeneous prolactin to *Oncorhynchus keta* (chum salmon) stimulated the mitogenic responses in the WBC cells. Sakai *et al.,* (1996) reported that prolactin enhanced the production of superoxide anion by WBC in rainbow trout. Moreover, lactoferrin, supplemented diets were reported to enhance the resistance against *A. hydrophila* in Nile tilapia (*Oreochromis niloticus*). In addition, innate and acquired immune activities such as respiratory burst, serum bacteriocidal activities bactericidal activities, serum lysozyme activities, and WBC counts of Nile tilapia was observed to increase against *A. hydrophila* following the lactoferrin diet feeding process (El- Ashram and El-boshy, 2008)

Animal derivatives

Ecteinascidia turbinate extract (ETE) when injected to eel (*Anguilla rostrata* enhanced the survival rate when challenged with *A. hydrophila* (Davis and Hayasaka, 1984). *Haliotis Discus hannai* extract (HDe) when intraperitoneally injected to rainbow trout significantly promoted the natural killer cell activities, phagocytosis and survival against *V. anguillarum* infection (Sakai *et al.,* 1991). In addition, Siwicki*et al.,* (1994) elucidated the production of superoxide anion, potential killing activities by macrophages and the lymphoblastic transformation of WBC under *in vitro* condition in rainbow trout after administration of partially heat treated extract derived from firefly squid (*Watasenia scintillans*).

Plant derivatives

Nowadays, considerable attention has been paid in the use of eco-friendly plant based products for the treatment and cure of different diseases. Traditionally, medicinal plants have been used from time immemorial to treat many diseases (Bhadauria*et al.,* 2002). The plant kingdom consists of divergent species with unique secondary metabolites that have significant properties such as antimicrobial activity, bacteriostatic activity, and chemotherapeutic functions (Purohit and Mathur, 1999). Currently, extensive

research and development has been has been commenced to determine the possibilities of using herbal medicines in fish disease management under the consideration that these products are often without side effects and are eco-friendly. Moreover, other factors such as, bioavailability, cost effective raw material and ease of processing has made the use of medicinal plants in aquaculture an added advantage (Harikrishnan and Balasundaram, 2005).

Several research reports have substantiated the role of medicinal plants in the enhancing the fish immunity. Administration of *Astragalus* extract to Nile tilapia for one week significantly enhanced the phagocytic activity and leucocyte counts (Yin *et al.,* 2006; Ardo*et al.,* 2008). In common carp, feeding of *Astragalus* extract showed enhanced cell mediated immune response including respiratory burst, phagocytic and lysozyme activities thereby increasing the survival of fish against *A. hydrophila*challenge (Yin *et al.,* 2009). Divyagnaneswari*et al.,* (2007) reported a decrease in fish mortality against *A. hydrophila* challenge and enhanced respiratory burst activity in tilapia after administration of *Solanum trilobatum* water extract.

Similarly, Dugenci*et al.,* (2003) recorded an enhanced phagocytic and respiratory burst activity of WBCs in rainbow trout after feeding with ginger extract. In another example, an experimental feeding trial in which carps were fed with diets supplemented with a combination of medicinal plants such as *Polygonum multiflorum*, *Astragalus membranaceus*, *Glycyrrhiza glabra*, and *Isatistinctoria* at a concentration of 0.5 % and 1 % for 30 days recorded significant increase in total serum proteins, respiratory burst and phagocytosis activity (Yuan *et al.,* 2007). Moreover, the immune parameters were significantly increased in large yellow croaker and common carps fed with a mixture of *Angelica sinensis* and *Astragalus membranaceus* supplemented diets (Jian and Wu, 2003; Jian and Wu, 2004). Similarly, Sahu*et al.,* (2007a; 2007b) recorded significantly increased lysozyme activity, superoxide anion production, bactericidal activity and reduced mortalities after *A. hydrophila*challenge in *Labeorohita* fed with *Allium sativum* and *Magniferaindica* incorporated diets. Kaleeswaran and Ilavenil, (2011) elucidated that administering diets supplemented with *Cynodondactylon*to Indian major carp *Catlacatla* enhanced the growth, immunity and resistance against *A. hydrophila*infection.In addition, *Zingiberofficinale* incorporated diets were observed to stimulate the nonspecific immune response and better survivability against *A. hydrophila* in Asia sea bass (*Latescalcarifer*) (Talpur*et al.,* 2013). In another report, Goshal*et al.,* (2016) elucidated enhanced growth an survivability of monosex Nile tilapia (*Oreochromisniloticus*) after experimental feeding of *Basellaalba* leaves and *Tribulusterrestris* seeds incorporated diets.

Fish immunity

The immune system of fishes are broadly classified into innate (non-specific) and acquired (specific/adaptive) immune system. Immune defence mechanism of fish against bacterial pathogens was studied in detail (Ellis, 1999). Earlier, the immunity was classified as two separate types and considered to act independently. But, research has shown that innate and adaptive immune system often work together. For example macrophages not only phagocytose the pathogens but secrete cytokines which in turn stimulates the adaptive immune response. Moreover, antibodies are synthesized by fish lymphocytes after being stimulated by the cytokines and essential complement components of innate immune system (Lydyard*et al.,* 2004).

Acquired (specific) immunity

Acquired immunity plays a major role in host immune system by eliciting cell-mediated immune mechanism (i.e., generating memory cells) which offers protection against recurrent microbial infections and humoral immune mechanism (i.e., T lymphocytes and Immunoglobulins) which includes specific membrane bound receptors enabling efficient elimination of the pathogen (Galindo-Villegas and Hosokawa, 2004; Romboute*t al.,* 2014). T-cells are further classified in to helper T-cells (T_h cells) and cytotoxic T-cells (T_c), which are functionally different. T_h cells stimulates immune response of B-lymphocytes resulting in a cascade activity by secreting short peptide signal molecules called cytokines. On the other hand, T_c eliminates intracellular pathogens and viruses by eliciting an apoptotic function (Secombes*et al.,* 2005). Soon after being induced by the cytokines of T_c the B-lymphocytes synthesize different classes of immunoglobulins (Ig) (Lydyard*et al.,* 2004).

Cytokines and its role in immunity

Cytokines refers to two Greek words namely cyto-cell and kinos-movement. These are regulatory proteins synthesised by immune cells that stimulate and regulate the immune cell functions (Thomson, 1994). These molecules play a vital role in maintaining the homeostasis of the immune response mechanism such as acute phase response, inflammation, and tissue repair (Ollier, 2004). Cytokines consists of three groups namely lymphokines secreted by lymphocytes, monokines produced by monocytes and interleukins (IL) secreted by leucocytes which stimulates other leucocytes. Recently with the aid of advanced molecular techniques , a number of cytokines genes from fish has been isolated and sequenced.

Innate (non-specific) immunity

Innate immunity provides the first line of defence for the host against pathogen attack, and prevents the establishment of infection followed by the activation of the acquired immune response. The innate

immune system has non-self-recognition mechanism which is regulated by proteins and/or receptors which recognize the germ line encoded molecular patterns (Magnadóttir, 2006; Lazado and Caipang, 2014). Such non-self-recognition molecules include LPS, polysaccharides, peptidoglycans and nucleic acids (Medzhitov and Janeway, 1998; Medzhitov and Janeway, 2002; Elward and Gasque, 2003).

Substituting fish meal with plant meal

Many investigations have been carried out to study the use of plant protein derived from seeds, leaves and other agricultural by products (Chavez, 1988; El-Sayed, 1999; Makkar&Becker, 1999; Siddhuraju*et al.,* 2000; Hossain *et al.,* 2001; Afuang*et al.,* 2003; Richter *et al.,* 2003). In salmons, replacing fish meal diet with many cheaper plant protein source such as peas and faba beans (Gouveia *et al.,* 1993; Pfeffer *et al.,* 1995), canola, rapseed (Stickney *et al.,* 1996), lupin (Glencross *et al.,* 2004; Burel*et al.,* 1998; 2000) and other plants (Watanabe and Pongmaneerat 1993; Morales *et al.,* 1994; Sanz *et al.,* 1994) was done to enhance the immunity and growth. However, complete incorporation of plant proteins in fish diet was reported to decrease the fish growth (Fontainhas- fernandes*et al.,* 1999; Glencross *et al.,* 2004). In addition, higher level of protease inhibitors, crude fibres and anti-nutritional compounds in feeds may affect protein digestibility resulting in decreased growth (Olli *et al.,* 1994; Vielma*et al.,* 2000; Al-Ogaily and Al-Asgah, 2003; Siddhuraju*et al.,* 2000). Soltan*et al.,* (2008) elucidated that substitution of plant meals at a level > 45 % led to significantly reduced feed intake, feed conversion ratio (FCR) and also reduced growth parameters such as weight gain, in Nile tilapia.

Moreover, marked decrease of FCR and weight gain was observed after complete replacement of fish meal with a mixture of plant protein in Nile tilapia (Sitjà-Bobadilla *et al.,* 2005). Conversely, when substitution of about 68 % of fish meal with different qualities of soybean meals and corn gluten resulted in absence of any significant changes in growth of Atlantic cod (*Gadusmorhua*) (Albrektsen*et al.,* 2006; Refstie*et al.,* 2006). Similarly, reports by Espe*et al.,* (2007) elucidated that complete (100 %) replacement of diets for *Salma salar*had no deterring effect on the growth and feed intake, provided if the amino acid profiles of the diet is well balanced. According to Lee*et al.,* (2002) complete substitution of fish meal with a mixture of plant proteins (cottonseed meal and soybean meal) and animal derived proteins showed enhanced growth rate and feed utilization in rainbow trout. However, when fish meal was replaced with < 50% of plant material significant effect on fish growth. Siddhuraju*et al.,* (2000) reported an enhanced growth in the moringa leaf extract at 5 % incorporated diet in the fresh water carps. On the other hand, when the plant mixture was fed for long duration it resulted in reduced FCR and weight gain in Nile tilapia, which was attributed to anti-nutrient compounds (alkaloids, lectins) present in the leaves Richter *et al.,* (2003).

Fournier *et al.,* (2004), evidently showed that 50 % fish meal replacement by lupin, wheat and corn gluten in the diets of juvenile turbot (*Psetta maxima*) showed no significant difference in the growth rate, whereas a 100 % replacement significantly reduced growth rate. On the other hand, tilapia and tilapia hybrids (*O. niloticus x O. aureus*) were reported with enhanced growth when fed with diets containing corn bi products and soybean meal as protein sources (Wu *et al.,* 1994; Twibell and Brown, 1998). Jia *et al.,* (1991) reported that incorporation of alfalfa in diets of Chinese blunt snout bream (*Megalobramaamblycephala*) increased feed intake. Certainly, Bilgin*et al.,* (2007) observed that hazelnut meal could replace 20% and 30% of dietary soybean meal without growth suppressing effects on specific growth rate, relative growth rate, feed efficiency, and survival. In contrast, Glencross *et al.,* (2004) recorded a reduced growth rate of rainbow trout when the concentration of yellow lupin level (0, 12.5, 25, 37.5 and 50%) was increased in diets.

Significance of the present study

Medicinal plants are widely used component of traditional medicines and it has been the cultural heritage of many people from diverse cultures and countries. Reports estimate that over 6,000 plants in India are employed in the traditional and herbal medicine, proclaiming 75 % of the medicinal need among the world countries (Pushpangadan*et al.,* 2001).The use of immunostimulants as an alternative component to chemotherapeutic agents, has gained prominence in fish disease control and it has become the major focus in the field of aquaculture over the past few decades. In this context, many research reports have emphasized on the use of medicinal plant products as potential therapeutic measures for modulating the immune response and specifically, on the use of herbs to prevent and control fish diseases (Galina *et al.,* 2009).

Generally, phytochemicals aid the immune system by improving the functions of lymphocytes, macrophages, NK cells (Craig, 1999). Agarwal and Singh (1999) published a comprehensive review of Indian medicinal plants and associated products shown to have immunostimulatory properties in mammalian models. Among them, remarkable reports with respect to aquaculture exist in *Ocimum sanctum, Phyllanthus emblica,Azadirachtaindica, Mentha piperita,* and *Solanum trilobatum* etc. Based on these literature studies and the well-established fact that medicinal plants can be used as immunostimulants to enhance the physiological, immunological and disease resistance of cultured fish, the present study aims to determine the immunostimulant activity of *Allium cepa* L. in the fresh water fish *Poeciliasphenops.*

OBJECTIVES

The present study was carried out to determine the immunostimulant activity of *Allium cepa*with the following objectives,

- To collect and process *Allium cepa* L. peels and formulate herbal feed at different concentrations.
- To determine the *in vitro* immunostimulatory activity of *Allium cepa* L. after dietary supplementation to *Poeciliasphenops*for 14 and 28 days.
- To examine the effect of *Allium cepa* L. dietary supplementation on the enhancement of growth, haematological and biochemical parameters.
- To study the relative expression of innate immune gene (IL-10) after experimental feeding trial.

METHODOLOGY

Collection and processing of plant material

The bulb part and peel of *Allium cepa* were collected from local vegetable market in Sundarapuram, Coimbatore, Tamil Nadu, India. Soon after collection the plant materials were dusted out of sand and other particulate matters and stored in cool dry place.

Bulbs and peels of *A. cepa* were thoroughly washed under tap water, shade dried for three week until all the moisture has been removed, coarsely powdered using mixer grinder and stored in an air-tight container for further use.

Experimental system, animals and diet formulation

*Poeciliasphenops*fingerlings with an average weight of 3.5 ± 0.5 g were procured from Induced carp spawning and seed rearing centre, Tamil Nadu, India Fisheries Development Corporation (TNFDC, Govt. of India), Aliyar, Tamil Nadu, India. The fingerlings were grown in recirculating tanks maintained using stringed bed suspended bioreactor (Kumar *et al.,* 2013b). The basal diet was formulated according to the diet formulation routinely used at TNFDC, Aliyar. Balanced basal diet without *A. cepa*supplement was used as control feed. The experimental diets were prepared by supplementing *A. cepa*(AC) to the basal diet at varying concentrations (1.5 g, 3.0 g, and 6.0 g / 100 g basal feed).

Proximate analysis of feed

The proximate analysis of the feed was evaluated based on the standard methods of the Association of Official Analytical Chemists (AOAC, 1990). Moisture content was determined gravimetrically in a hot air oven at 100 ± 10 ℃ for 24 h. Crude protein content was determined by the Kjeldahl method. Crude lipid was estimated by extraction with petroleum ether (boiling point: 40–60 ℃) in Soxhlet apparatus (Borosil, India). After extraction of the lipid the defatted samples were used for estimation of crude fibre. Ash content was estimated by igniting samples in a muffle furnace (Model No. KSSMF003, Metrolabs, India) at 50 ± 5 ℃ for 10 h.

Experimental design and feeding trials

For growth performance and immune response trials, the experimental facility consisted of 12 tanks (100 L each). *Poeciliasphenops*fingerlings were randomly distributed into cultivating system in triplicates (at a density of 25 fish/tank). The feeding trial was performed for four treatment groups:

Control group T_0 – were fed with basal diet.

Experimental group T_1 –was fed with 1.5 % AC mixed diet.

Experimental group T_2 –was fed with 3.0 % AC mixed diet.

Experimental group T_3 – was fed with 6.0 % AC mixed diet.

The fish were hand fed twice daily (9:00 and 17:00) at 5 % body weight during the feeding trial for 14 and 28 days. Ten fish were selected for growth analysis whereas six fish were randomly sampled from each respective groups at 14 days intervals (i.e., 14 and 28 days) for haematological and biochemical assays. For bacterial challenge analysis, six fish were cultivated in non-circulating system, with three-day interval water exchange. All fish were fed with their respective diets before challenge study using *A. hydrophila*. Post challenge study was continued up to 10 days. At the end of the post challenge the disease resistance and relative percentage of survival was estimated for all the groups.

Growth analysis

Growth analysis was performed according to the method of Olmedo sanchez*et al.,* (2009). Growth rate was recorded after 14 and 28 days by randomly weighing 10 fish from each group in triplicates. Each fish was caught from respective tank and weighed using an analytical balance (Citizen CX 304, India). The fish were carefully returned to its appropriate tank after measurement. The growth performance of fish was evaluated on the basis of Initial body weight (IBW), Final body weight (FBW), Live weight gain (LWG), feed conversion ratio (FCR), protein efficiency ratio (PER), and specific growth rate (SGR) using the following formulae:

Live Weight Gain (LWG) = Final body weight (g) - Initial body weight (g)

Specific growth rate (SGR) = [(Ln Final weight-Ln Initial weight) ÷ No of days in trial] × 100

Feed conversion ratio (FCR) = Feed given (dry wt) ÷ Weight gain (wet weight)

Protein efficiency ratio (PER)=wet weight gain by fish (g) ÷ Protein intake (g)

Haematological parameters

Blood was collected after experimental feeding trial and was immediately used to assess the number of red blood cells (RBC) and white blood cells (WBC) (Blaxhall and Daisley, 1973). Haemoglobin content was determined by following the cyanomethemoglobinmethod of Oser and Hawk, (1965). Differentiation of blood cell was performed according to Sarder*et al.,* (2001). The fish was anesthetized by administering clove oil (Himedia, India) (100 µl /L) of water before collecting blood samples from fish. Blood was drawn from caudal vein of fish by using 1 ml hypodermal syringe and 24 gauge needles which were pre- rinsed with 2.0 % EDTA solution before use. The collected blood was transferred to the EDTA treated test tubes and shaken well in order to prevent clotting of blood. Serum was collected without using anticoagulant and was separated from blood by keeping the tubes in slanting position for about 2 h and thereafter it was centrifuged at 1000 × *g* for 15 min at 4 ℃, followed by collection of straw coloured serum and stored at – 20 ℃ for further analysis.

The collected blood was immediately used to assess the number of red blood cells (RBC) and white blood cells (WBC) by using a haemocytometer slide (Improved Neubauer type; Merck, Lutterworth, U.K) at a magnification of x 400. Thus, blood was diluted to 10^{-2} and 10^{-3} in 1X PBS at pH 7.2 (Blaxhall and Daisley, 1973). Haemoglobin (Hb) content was determined by following the cyanomethemoglobin method of Oser and Hawk, (1965). Briefly, 20 µl of the blood (stored in room temperature for at least 10 min) was mixed with 5 ml of Drabkin's solution (Himedia, India) and the absorbance was measure at 540 nm. The concentration of Hb was determined using the formula,

In order to differentiate the blood cells, blood films from triplicate samples were prepared on clean microscope slides, fixed in 95 % methanol for 5 min and air dried. The slides were stained with Giemsa's stain (Himedia, India) for 20 min according to Sarder*et al.,* (2001) and observed at a magnification of × 400 using light microscope (Nikon eclipse, MV-E200 series).

Phagocytic activity

Phagocytic activity (PA) was determined using *Staphylococcus aureus* MTCC 96 (MTCC, Chandigarh, India) as described by Anderson and Siwicki, (1995). A sample (0.1 ml) of blood leucocyte obtained from individual fish was placed in a micro titre plate well, 0.1 ml of *S. aureus* (1×10^7cfu/ml) cells suspended in PBS (pH 7.2) were added and mixed well. The bacteria-blood leucocyte solution was incubated for 20 min at room temperature. 5 ml of this solution was taken on to a clean glass slide and a smear was prepared. The smear was air dried, then fixed with ethanol (95 %) for 5 min and air-dried. The air-dried smear was stained with 7 % Giemsa for 10 min. Two smears were made from each fish. The total of 100 neutrophils and monocytes from each smear were observed under the light microscope (Nikon Eclipse-E200, Japan) and the numbers of phagocytising cells were counted. Phagocytic activity (PA) percentage was calculated as follows,

PA % = (Number of phagocytizing cells × 100) / Total number of phagocyte cells counted

Determination of humoral immune response

The total protein content of the serum was determined according to Lowry method (Lowry *et al.,* 1951) using bovine serum albumin (BSA) as the standard. Briefly, 200 µl of BSA working standards were taken in five test tubes and made up to 1 ml using distilled water. The tube containing 1 ml distilled water served as control. Then, 4.5 ml of reagent A was added to each tube and incubated for 10 min. After incubation, 0.5 ml of reagent B was added and incubated for 30 min. Then the absorbance was measured at 660 nm and the standard graph was plotted. The total protein content of the unknown serum sample was estimated by interpolating the absorbance of the unknown sample on to the standard curve and the concentration was determined.

***A. hydrophila*challenge and Histological studies**

After each experimental feeding period each fish was exposed to *A. hydrophila* according to Jha *et al.,* (2007). Histological studies were performed according to Kaleeswaran*et al.,* 2012). After immunization with *A. hydrophila*, the experimental fish (120 days batch) organs samples such as kidney, liver and intestine were taken at 7^{th} day after immunization. The organ samples were removed and fixed in 10 % neutral buffered formalin, embedded in paraffin and stained with hematoxylin and eosin (H&E) for optical examination. Micrograph of the organs were taken from the paraffin sections at a final magnification of × 200 using a Nikon Eclipse E-200 microscope. For each group, micrographs originating from six fishes (n = 6) were analysed (Kaleeswaran*et al.,* 2012).

RNA extraction from head kidney and reverse transcription

Total RNA was extracted using 50-100 mg of kidney tissue using TRI reagent (Sigma, USA) as per manufacturer's protocol. The RNA concentration in the sample was quantified by measuring the absorbance at 260 nm using Bio photometer Plus (Eppendorf, India). The purity of the samples was analyzed by measuring the ratio of OD 260 nm/OD 280 nm. The samples having OD ratio of 1.8 - 2.0 were taken for the cDNA synthesis. The resulting RNA product was run on a 0.8 % agarose-MOPS-formaldehyde gel. Briefly, 0.8 g agarose was added to 80 ml distilled water followed by the addition of 10 ml 10X MOPS buffer (refer appendix). The mixture was allowed to melt using microwave oven. After slight cooling, 10 ml formaldyde was added to mix after. The mixture was poured into a 5.7 cm × 8.3 cm gel (W x L), and the edges were sealed with autoclave tape. A comb with 8 wells was inserted into the gel to form the sample slots. After the gel was completely set, the comb was removed and enough 1X MOPS buffer was added such that the gel is immersed. About 20 µg of RNA samples were then heat treated in a water bath at 65-70 ºC with double the volume of loading buffer for 10- 15 min and cooled on ice. The samples were given a brief spin and loaded on the gel. Electrophoresis was carried out for 45 min at 150 V and photographs were taken using a gel documentation unit (E-gel imager system, Thermo fisher scientific, India).

Total RNA (2 µg) was used for first strand cDNA synthesis using Revert aid first strand cDNA synthesis kit (Thermo scientific, USA) in thermo cycler (Eppendorf, Master cycler Pro S, USA). RNA was incubated with 1 µl of oligo $(dT)_{18}$ (20 µM) at 65 ºC for 5 min followed by 4 ºC for 5 min for primer annealing. After this, the following components were added to the reaction in order: 4 µl of 5X M-MuLV-RT buffer, 1 µl of Ribolock RNase inhibitor (20 U/µl), 2 µl 10 mM dNTPs, 1 µl of RT enzyme, 5 µl DEPC water. The reagents were gently mixed and incubated in the order, 5 min at 25 ºC, 1 h at 42 ºC, and 10 min at 70 ºC followed by chilling at 4 ºC. The synthesized cDNA was stored at -20 ºC for further use.

Primer design and optimization of PCR conditions

Primer design for cytokine gene (IL-10 and β-actin) expression studies was designed according to Kadowaki *et al.,* (2013) using the Primer 3 software in order to eliminate the primer dimer formation and increase the probability of getting the best PCR product with SYBR green fluorescence. Initial parameters for selecting the primers were as follows: Product size – 100 to 350 bp, Primer size – 20 ±2 bp, GC content – 50 ±5 %, T_m – 60 ± 2 ºC, Self-complementarity – 0 (Table 1).

Table 1. Oligo nucleotide primer sequences for semi quantitative PCR

Candidate Gene	Type	Primer sequence (5 ′ to 3 ′)
IL-10	FP	ACC TGC ACA TTG CTA CAG CA
	RP	CCC GCT TGA GAT CCT GAA AT
β-actin	FP	GGT CAT CAC CAT TGG CAA TG
	RP	CAG GGA TGT GAT CTC CTT CT

PCR conditions were optimized according to the laboratory conditions and primer annealing temperature was standardized.

Statistical analysis

All experiments were conducted in triplicates. The statistical analysis was performed using SPSS (V 21, Chicago, USA). Values were expressed as mean ± S.E. Statistical tests such as One way ANOVA with Tukey's post hoc analysis was used to test the immunostimulant activity, paired sample T test and pair wise fixed reallocation randomisation test in REST software was used to test the PCR data, The values of the data were considered significant if $p \leq 0.05$.

RESULTS

Proximate composition of the diets used in the study

The proximate analysis was performed to determine the nutritional composition of the diets used in the experiment. Results showed that nutritional factors such as crude protein, lipid fibre and ash contents showed a slight difference in the *A. cepa*supplemented feed as compared to the basal feed composition. Moisture content was observed to be at an average of 6 % on all the formulation suggesting that the feed contained minimal moisture content to prevent microbial contamination as per AOAC guidelines (Table 2).

Table 2. Feed formulation and proximate composition of experimental diets.

Materials	Formulation (g/100 g on dry matter basis)			
	Control (T_0)	T_1	T_2	T_3
Rice bran	45	45	45	45
Groundnut oil cake	20	20	20	20

Maize	10	10	10	10
Finger millet	10	10	10	10
Pearl millet	10	10	10	10
Agrimin forte*	5	5	5	5
Allium cepa (AC) powder	0	1.5	3.0	6.0
Proximate compositions[a]				
Crude Protein	35.13	35.55	35.64	35.74
Crude Lipid	11.47	11.83	11.85	11.9
Crude fiber	2.23	3.06	3.07	3.13
Ash	9.3	9.52	9.6	9.3
Moisture	6.3	6.05	6.48	6.2

Abbreviations of groups:
T_0: Control feed
T_1 : feed mixed with 1.5 % *A. cepa*
T_2: feed mixed with 3.0 % *A. cepa*
T_3: feed mixed with 6.0 % *A. cepa*
[†]Moisture is expressed as percentage of fresh weight; crude protein, crude lipid, and ash are expressed as percentages of dry matter. Each datum is a mean from three separate determinations.

Growth analysis

The effects of *A. cepa* supplemented diets on growth response are provided in Table 3 and Table 4. There was a steady increase in the weight for after fourteen days in all fish groups during the whole experimental period. The live weight gain (LWG) of the experimental groups were significantly ($p \leq 0.05$) different as compared to control over the entire feeding period. Significantly higher LWG was observed in fish fed with 3 % AC (T_2) supplemented feed than other groups. The results indicate that the specific growth rates (SGRs), feed conversion ratios (FCRs) and protein efficiency rates (PERs) of the experimental groups were significantly ($p \leq 0.05$) different as compared to control group on 14 and 28 days feeding period. However, statistical analyses showed no significant difference in SGR, FCR and PER of experimental and control groups after feeding period.

Table 3.Growth parameters of *P. sphenops* fingerlings fed with different levels of AC diets

Groups	IBW (g)	FBW (g)		LWG (g)	
		14 days	28 days	14 days	28 days
T_0	3.5±0.13 [a]	4.1±0.3 [a]	4.3±0.8 [a]	0.6±0.2 [a]	0.8±0.1 [a]
T_1	3.1±0.15 [a]	5.4±0.3 [b]	8.2±0.4 [ab]	2.3±0.2 [b]	5.1±0.4 [b]
T_2	3.4±0.10 [a]	5.2±0.4 [c]	7.1±0.4 [c]	1.8±0.3 [c]	3.7±0.4 [c]
T_3	3.2±0.08 [a]	5.2±0.2 [bc]	7.5±0.8 [bc]	2.0±0.2 [bc]	4.3±0.9 [bc]

Abbreviations :

AC diet : *A. cepa* supplemented diet.
IBW : initial body weight
FBW : final body weight
LWG : live weight gain.

Data are expressed as mean ± SE, n= 10.Mean values with different superscripts in same column were significantly different ($p \leq 0.05$) from the control.

Table 4. Growth parameters of *P. sphenops* fingerlings fed with different levels of AC diets.

Groups	SGR (%/day)		FCR		PER	
	14 days	28 days	14 days	28 days	14 days	28 days
T_0	2.0±0.03 [a]	1.3±0.02 [a]	1.0±0.03 [b]	1.2±0.04 [b]	0.40±0.01 [a]	0.4±0.02 [b]
T_1	2.2±0.05 [b]	1.5±0.02 [b]	0.9±0.03 [a]	0.9±0.03 [a]	0.33±0.01 [b]	0.3±0.01 [a]
T_2	2.3±0.04 [b]	1.5±0.01 [b]	0.8±0.02 [a]	0.9±0.02 [a]	0.31±0.01 [b]	0.3±0.01 [a]
T_3	2.2±0.02 [b]	1.5±0.02 [b]	0.8±0.01 [a]	0.9±0.03 [a]	0.32±0.05 [b]	0.3±0.01 [a]

AC diet :*A. cepa*t supplemented diets.
SGR (%) : specific growth rate
FCR : feed conversion ratio
PER : protein efficiency ratio.

Data are expressed as mean ± SE, n= 10.Mean values with different superscripts in same column were significantly different ($p \leq 0.05$) from the control.

Haematological parameters

The results of the haematological analysis showed a significantly ($p \leq 0.05$) different RBC level in the experimental groups T_2 and T_3 , over the entire feeding period as compared to the control (T_0) (Figure 1). The RBC count of T_1 was significantly different from control on 14 days experimental period. Significantly higher RBC count was recorded in group T_2 whereas lower RBC count was recorded in control. WBC counts were significantly ($p \leq 0.05$) different in experimental groups over entire feeding period as compared to control (T_0). Significantly higher WBC count was observed in T_2 among other experimental groups while

lower WBC count was observed in T_0 (Figure 2). Moreover, the haemoglobin level of groups T_2 and T_3 varied significantly ($p \leq 0.05$) over the feeding period as compared to control (T_0). The haemoglobin content of T_2 and T_3 groups varied significantly ($p \leq 0.05$) as compared to T_0 on all feeding period (Figure 3). Higher haemoglobin level was observed in T_2 as compared to other experimental groups over 14 to 28 days feeding period.

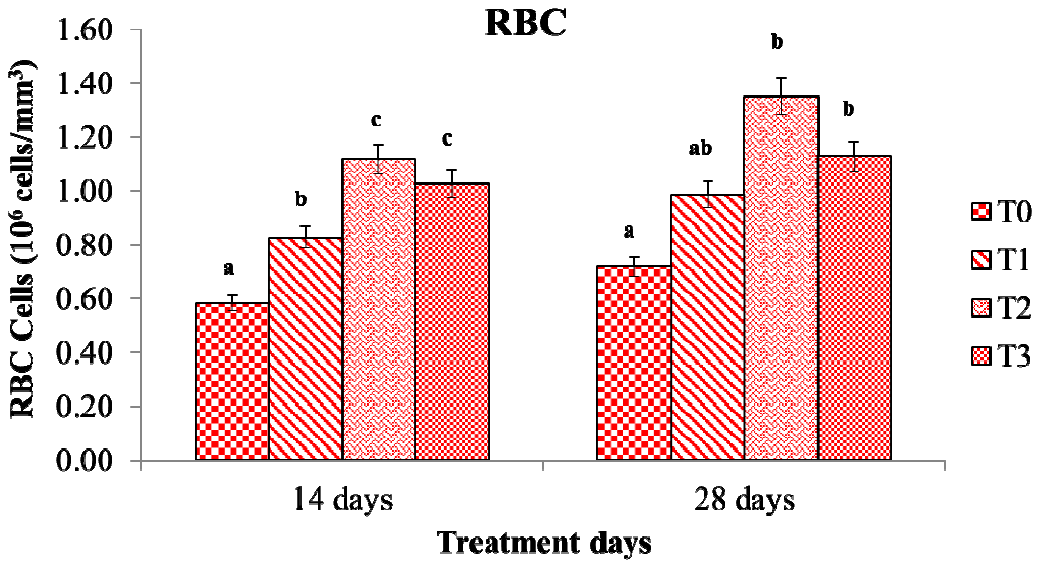

Figure 1. Haematological parameters (RBC) of *P. sphenops*fed with *A. cepa*diets at different levels for 14 and 28 days.

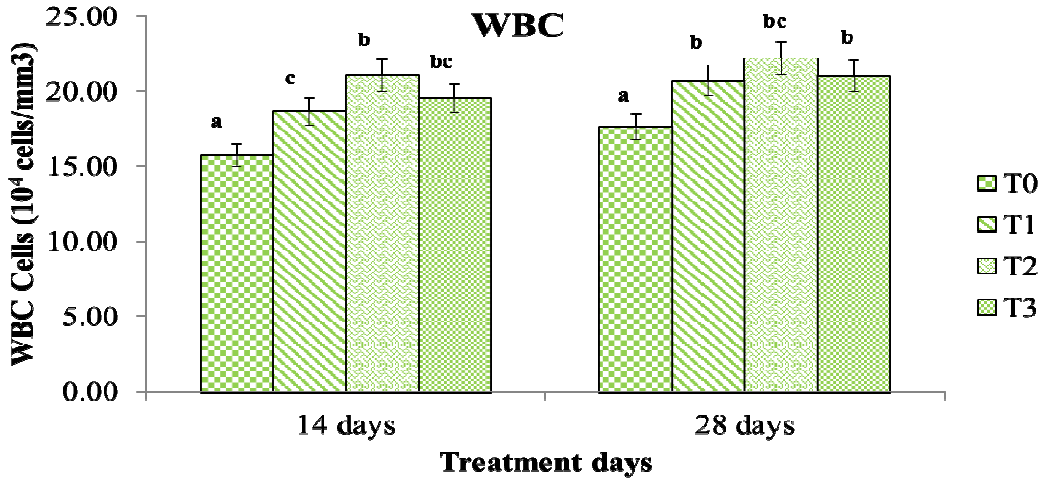

Figure 2. Haematological parameters (WBC) of *P. sphenops*fed with *A. cepa*diets at different levels for 14 and 28 days.

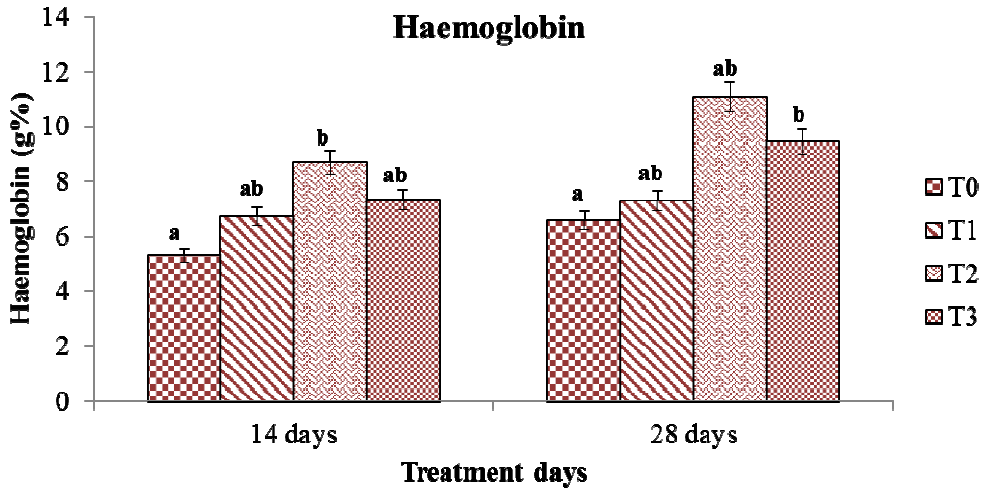

Figure 3. Haematological parameters (Hb) of *P. sphenops*fed with *A. cepa*diets at different levels for 14 and 28 days.

Estimation of Total Serum protein

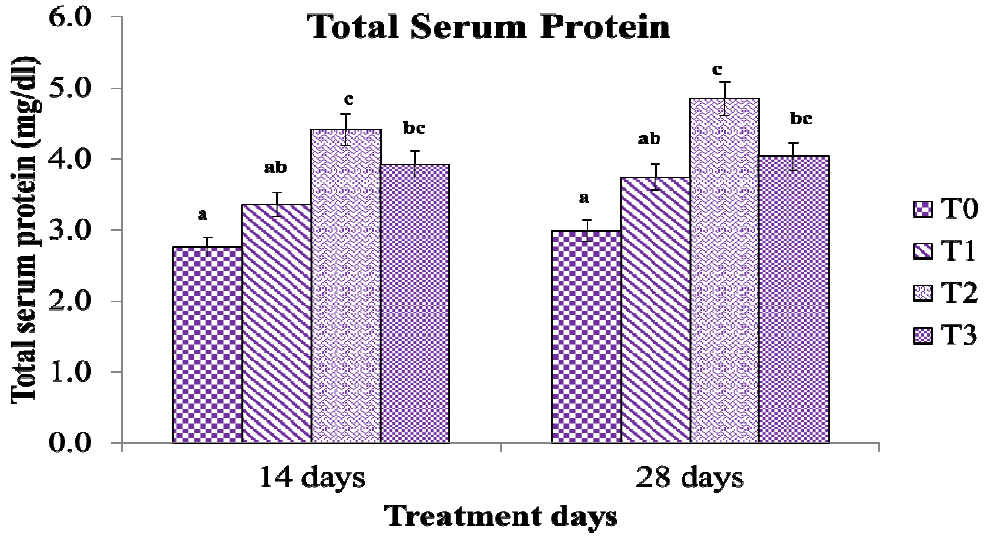

Figure 4. Determination of humoral immune response factors of *P. sphenops*fed with different concentration of *Allium cepa* supplemented diet for different experimental feeding days.

In relation to different serological parameters the total serum proteins (TSP), was determined. Total serum protein level was significantly different ($p \leq 0.05$) in experimental groups T_1,T_2, and T_3 over the entire feeding period as compared to control group (T_0). Significantly higher total protein content was observed in T_2 whereas lower serum protein level was observed in control group (Figure 4).

Phagocytic Activity Percentage

Phagocytic activity was significantly ($p \leq 0.05$) different in the experimental groups (T_1, T_2 and T_3) as compared to control (T_0) throughout feeding period (Figure 5). Higher phagocytic activity percentage (52.41 %) was observed in groups T_2 as compared to T_0 after 28 day feeding period.

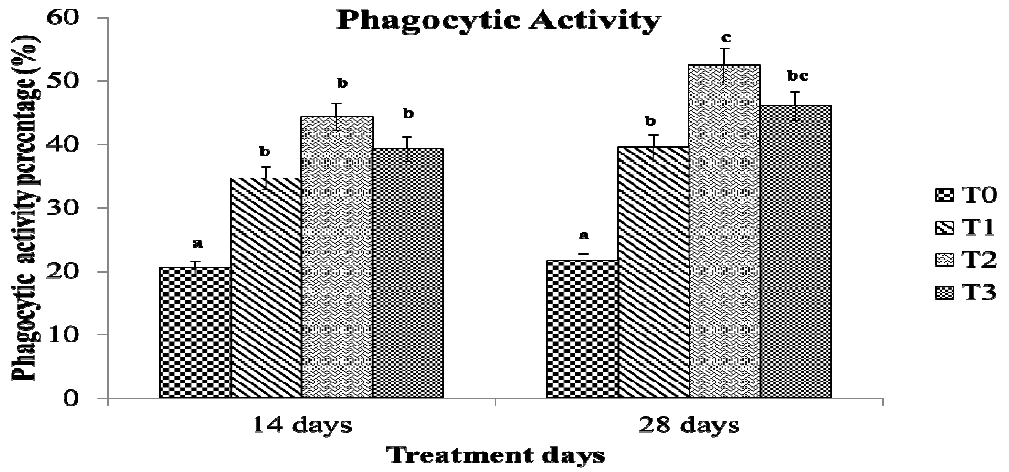

Figure 5. Phagocytic activity percentage of *P. sphenops*fed with different concentration of *Allium cepa* supplemented diet for different experimental feeding days. Data are expressed as mean ± SE; n = 6. The mean values with different superscripts are significantly ($p \leq 0.05$) different from control among groups.

***Aeromonas hydrophila* challenge and Histopathological studies**

The histological studies were performed to analyze the changes in the tissues samples *P. sphenops*fed with different concentration of *A. cepa* supplemented diet, before and after the *A. hydrophila*MTCC 1739 challenge. Moreover, this technique was aimed at determining the changes in the internal organs such as intestine and liver after 28 days administration of experimental feed.

Intestine

The histological preparations of *P. sphenops*intestines fed with basal diet (T_0) and *A. cepa* supplemented diet (T_1, T_2, and T_3) showed minor noticeable alterations in the intestinal walls. The lumen, villi, brush border cells, muscularis mucosa, sub mucosa and columnar absorbing cells appeared intact in all the groups before pathogen exposure. However, a change in the intestinal morphology was observed in all the groups after pathogen exposure which included the erosion of serosa of brush border cells, loosened lumen, reduced sub mucosal space, and modified morphology of columnar absorption cells with shrinkage and deformation. Such characteristics were well pronounced in the intestinal tissue of T_0 whereas it was observed to be less prominent in the intestinal tissues of T_1, T_2, and T_3. In addition, an increase in the

globular cells filled with vacuoles were observed in the intestinal walls of all the groups after pathogen exposure which was evidently less in the samples before pathogen exposure (Figure 6).

Liver

The liver cells showed normal hepatic cells with circumvented edges with normal connective tissues and with moderate hepatic mass granulation in the control samples (i.e., before pathogen exposure). Prominent central hepatic vein was observed in the cross section of the liver tissues taken from all the fish groups. The liver samples after pathogen exposure was observed with degenerated hepatocytes, narrowed Hepatic vein, aggregated inflammatory cells (melanomachrophage), karyomegaly, karyolysis, hepatic focal necrosis, and irregular hepatocellular arrangements. However, the degree of such characteristics were less and near normal appearances were observed in AC supplemented diet fed fish groups (T_1, T_2, and T_3) as compared to the basal diet fed fish (T_0). Also, moderate to heavy extravasation of inflammatory cells were observed near the hepatic portal veins of T_1, T_2, and T_3 test liver samples (Figure 6). In addition, hepatic tissues of the basal diet fed fish (T_0) showed a spongiform mass with scattered appearance with evident focal necrosis where as such conditions were not observed in the experimental diet fed fish liver tissues after pathogen exposure.

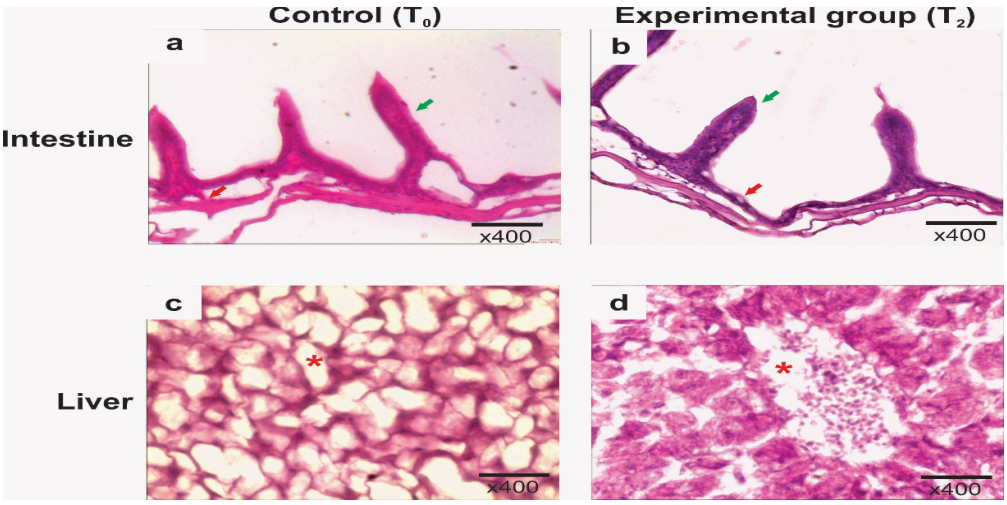

Figure 6. Light micrograph (× 400) of a, b) intestine and c, d) liver of *Poeciliasphenops* (T_0 and T_2) after 28 days experimental feeding of *A. cepa* supplemented diet. (Inset: Red Arrow head – inflammation of mucous muscularis leading to globular cells, Green Arrow head – brush bordered appearance of microvilli, Red asterisk – neutrophil intrusion).

RNA isolation and cDNA synthesis

The total RNA was extracted from the tissues of immunized fish from each group after twenty eight days experimental feeding trial. The extracted RNA was checked for its purity and evaluated for it quality by electrophoresis on agarose gel (Figure 7). The RNA samples showed two dense band with smear pattern on all the samples indicating the presence of 28S and 18S rRNA. Moreover a faint band pattern was observed which could be attributed to the 5S rRNA of the sample. The RNA samples consisting of 2.0 purity was then reverse transcribed into cDNA and then further subjected to gene specific amplification using semi quantitative PCR. The results of the gene specific PCR revealed an amplicon of approximately 330bp in the control and experimental group samples as along with the amplification of β-actin internal control.

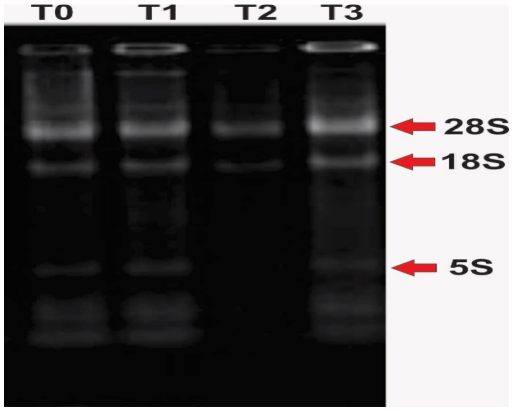

Figure 7. rRNA products extracted from fish fed with *A. cepa* supplemented diet for 28 days on 1 % agarose-MOPS- formaldehyde gel with 28S, 18S and 5S bands patterns.

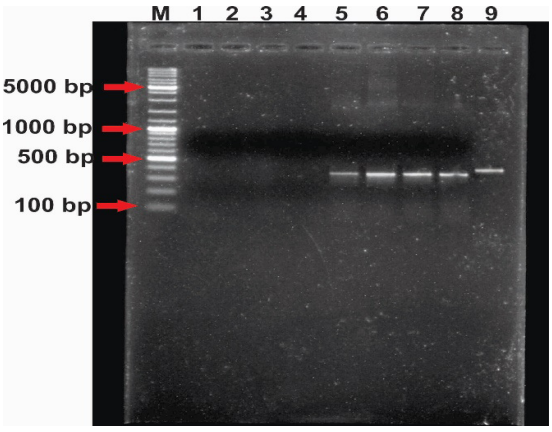

Figure 8. IL-10 amplicons of fish fed with different concentration (T0 to T3) of *A. cepa* supplemented diet for 28 days on 1 % Agarose gel.(M-1Kb DNAladder, Lane 1 to 4 – Negative control of groups T0 to T3 respectively, Lane 5 to 8 – IL-10 amplicon of ~300 bp and Lane 9 – β-actin internal positive control).

Following the semiquantitative PCR analysis the densitometric analysis of the gene speicifc amplification revealed that the expression level of IL-10 was significantly ($p \leq 0.5$) higher (2.2 fold) in the T2 group as compared to the other experimental groups with reference to beta actin internal control.

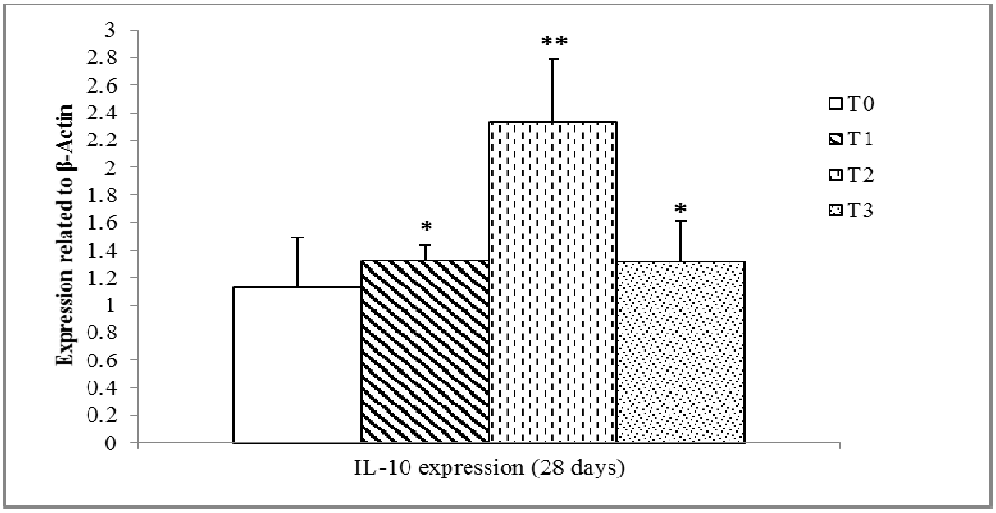

Figure 9. Densitometric analysis of IL-10 gene expression with reference to β-actin internal control. (*p≤0.05; **p≤0.01).

DISCUSSION

The results of this study showed the potential effect of *A. cepa*as a growth enhancer and stimulated fish appetence. Our results showed that, fish which received 3 % AC mixed diets exhibited increased weight gain from 14 to 28 days feeding period. However, no significant difference in SGR, FCR, and PER was observed between control and experimental groups at 28 days feeding period. This finding elucidated that incorporation of *A. cepa* in diets meet the optimal value in *P. sphenops*. Analysis of final body weight and weight gain showed that application of 3 % AC in total diets enhances the optimal growth of the fish. Similar requirement was recorded in *C. carpio*and *C. mrigala* by Darisini*et al.,* (2014), who reported that low concentration of dietary herbal supplement could influence a better growth in fresh water carps. It is suggested that 3 % AC supplementation in total diet is the optimal dosage for this fish. This may be due to difference in the individual fish nutrient acquisition, digestibility and feeding quantity. Besides, it has been demonstrated that factors such as species, age, size, and stress determine the appropriate usage of plant dietary supplements (Bendiksen*et al.,* 2003). Further several herbal supplements are reported to stimulate appetite and enhance weight gain when they are incorporated to cultured fish (Talpur*et al.,* 2014). It is hypothesized that higher dosage of herbal diet supplements has resulted in stimulation of enzyme inhibitors,

as most plants contain inhibitors to protect their major components from accidental degradation (Francis *et al.,* 2001).

Anti-nutrient substances present in plants such as protease inhibitors, amylase, lipase, tannins, saponins, lectins and antivitamins has been reported to cause disturbance in the gastrointestinal tract (Jobling, 2011). These conditions may have affected the feeding efficiency and digestive process leading to lack of complete nutrient utilization. Although no specific experiment was carried out to determine the anti-nutrients and inhibitors of *A. cepa*, our results revealed that different percentage of AC play a major role for the growth and feeding efficiency in experimental fish. The proximate composition of experimental and control diets were similar to diet composition of Darisini*et al.,* (2014) and contained protein, lipid, and fibre. As the bioactive principles of *A. cepa*has been cited to contain essential alkaloid, phenols, and other nutrient contents, it could be reasonably hypothesized that the bioactive compounds present in *A. cepa* supplemented diet may have enhanced fish health by stimulating innate immunity.

In the present study, dietary administration of AC enhanced the haematological indices of fish. Observations revealed that fish fed with 3 % AC supplemented diet showed significantly higher RBC levels as compared to other experimental groups over entire feeding period. However, no significant difference in RBC was observed in T_2 and T_3 as compared to control group (T_0) after 28 days of feeding period. The WBC level showed a significant difference in fish fed with AC diet as compared to control. Higher WBC level was observed in 3 % AC diet fed fish as compared to other groups. Moreover, the results indicated steady increase in the WBC with the feeding period. Herbal immunostimulants can affect the haematological parameters of fishes (Choudhury *et al.,* 2005; Talpur and Ikhwanuddin, 2012). Significant enhancement in haematological parameters may be due to enhanced erythropoietic centres in (kidney/spleen), decreased erythroclasia and enhanced Fe^{++} metabolism. Enhanced RBC and WBC counts following the AC diet feeding indicate the immunostimulant effect and anti-infection properties of onion. These results corroborate with the findings of Dina Rairakhwada*et al.,* (2007)and Talpur and Ikhwanuddin (2013), who reported an increase in RBC and WBC count after feeding levan to *Cyprinus carpio*and ginger supplemented feed to *L. calcarifer* fishes respectively.
In addition, Misra*et al.,* (2006a) reported an increase in RBC and WBC of rohu following feeding trial with herbal diets for 60 days. In the present study the experimentalgroups demonstrated a significant increase in haemoglobin content compared to control on the whole feeding period. Haemoglobin content in 3 % AC (T_2) diet fed fish increased significantly, followed by T_3 and T_1 as compared to control, during the whole feeding period. Significant enhancement in Hb content indicated that oxygen supply had increased and thus improved the well-being of fish (Talpur and Ikhwanuddin, 2012).

The results of our study showed higher phagocytic activity in AC diet fed fish groups compared to control group. In this study 3 % AC diet fed fish showed significantly higher phagocytic activity on all feeding period compared to other groups, which evidently indicated that AC diet supplement, enhanced the non-specific immunity of fish. Our results are in accordance with the report of Arulvasu*et al.,* (2013), who observed significantly increased phagocytic activities after feeding *C. catla* with ginger. Phagocytosis is an important defence mechanism in fish against bacterial pathogens (Sahu*et al.,* 2007; Nya and Austin, 2009).

A significant increase in total serum protein was observed in all experimental groups compared to control. Increase in serum total protein was reported in *C. catla* fed with ginger (Arulvasu*et al.,* 2013). Similar results were reported in *C. carpio* fed with *M. piperita*(Abasali and Mohamad, 2010)and *Latescalcarifer*fed with ginger (Talpur and Ikhwanuddin, 2013). Stronger non-specific immune response is associated with marked increase in the total protein, albumin and globulin levels of fish (Rao *et al.,* 2006; Sahu*et al.,* 2007; Nya and Austin, 2009).

In the present study, histopathological analysis of *P. sphenops*intestine and liver tissues indicated normal morphology in groups (T_0 and T_2) before *A. hydrophila*exposure. This resultscould be attributed to the non-toxic nature of the herbal supplement used in the present study. Similar results were observed by Kaleeswaran*et al.,* (2012) in *C. catla* after administration of *Cynodondactylon*supplemented feed.

In the present study, MMC was evidently observed in the epithelium, lamina propria and tunica serosa of the intestine of all the groups (T_0 and T_2) after pathogen exposure. Our findings were in agreement with that of Fournier-Betz *et al.,* (2000) who studied the immunoglobulin aggregation in the gut associated lymphoid tissues in turbot *Scophthalmus maximus* and reported higher aggregation of MMC in the epithelia of intestinal region. The histological observation of liver in the present study showed varying degree of morphological changes in the hepatic tissues of *P. sphenops* after infection with *A. hydrophila*. Here also, MMC was evidently observed significantly higher near the hepatic portal vein of T_2 as compared to T_0. Although, liver is not directly involved in the immune regulation of fish, it is the site of interleukin expression during the pathogen invasion and many reports have elucidated the significant expression of IL-10 in the liver which could be correlated with our report to support the hypothesis that long term feeding of *A. cepa*supplemented diets have enhanced the immunity of *P. sphenops* thereby promoting the MMC aggregation and reducing the severity of the infection (Sangrador-Vegas *et al.,* 2000; Subramaniam *et al.,* 2002).

In the present study, gene expression varied with dose of AC supplementation and time duration of experimental feeding study. In the densitometry analysis of the PCR products significant IL-10 gene expression was observed in all the experimental groups which received AC supplemented diet fed fish after 28 days. Many immunostimulants have been reported to induce the cytokine gene expression of fish immune system and increase their resistance against pathogens. For instance, Gioacchini*et al.,* (2008) reported a significant higher cytokine gene expression in the liver tissues of rainbow trout following the administration of Ergosan thereby elucidated its role in enhancing immune response. In another report, Yuan *et al.,* (2008) studied the expression of the immune response genes in head kidney, gill and spleen of common carp injected with *Astragalus* polysaccharide. Ergosan has been shown to exert a positive effect on liver cytokine (IL-1ß, IL-8 and TNF-α) gene expression in rainbow trout leading to a significantly higher expression, thus indicating a role in stimulating the innate immune response (Gioacchini*et al.,* 2008). In addition, Watanuki*et al.,* (2006) elucidated the up-regulation of IL-1β and TNF-α genes in common carp orally fed with *Spirulina plantensis*although there was down-regulation in IL-10 gene. Similarly, an increase in the complement reactive protein with antibody specificity to human IL-10, IL-12, IL- Iß and TNF- α was observed in Nile tilapia after oral administration of ß-1, 3-glucan (Chansue*et al.,* 2000). The cytokine IL-10 exhibits anti-inflammatory function and are regulated by multiple immune actors (Savan*et al.,* 2003). The primary function of IL-10 is to negate the pro-inflammatory cytokines (IL-1β, TNFα and IFN-γ) response and prevent tissue damage (Moore *et al.,* 2001).

SUMMARY

The present study evaluated the *A. cepa*(L.) bulb and peel powder for its immunostimulant properties in a bioprospective approach. The onion was subjected to feed formulation and studied for its *in vitro* immunostimulant properties in fish by using the *P. sphenops*as animal model. In this study, the *P. sphenops*fingerlings were subjected to experimental feeding study for 14 and 28 days in which the fingerlings were fed with different concentration of 1.5 % (T_1), 3 % (T_2) and 6 % (T_3) AC supplemented diet, while the fishes fed with basal diet was used a control (T_0). In order to elucidate the immunostimulant property of *A. cepa* supplemented diet on the immune response of the fingerlings after experimental feeding at molecular level, the fish were immunized with heat killed *A. hydrophila* and subjected to cytokine gene (IL-10) analysis of fish tissue using semi quantitative PCR technique.

- Dietary supplementation of *A. cepa* and experimental feeding analysis elucidated that 3 % AC supplemented diet was effective in significantly enhancing the growth parameters (FBW, LWG,

SGR, FCR and PER), cellular immune parameters (PA, Respiratory burst), humoral immune parameters (RBC, WBC, Hb, and Total protein) as compared to the control irrespective of the feeding period (14 and 28 days).

- Histopathological analysis evidently showed no significant changes in the internal organs (intestine, kidney and liver) of fish following the experimental feeding elucidating non-toxic nature of the *A. cepa*. In addition, the histopathological studies elucidated a near normal appearance of internal organs (intestine, kidney and liver) of fish after pathogen challenge, with higher melanomacrophagecentres as compared to the control group elucidating the enhanced immunity in the experimental group fishes.
- Molecular analysis of the candidate cytokine genes revealed a significant expression of IL-10 in 3 % (T_2) AC supplemented fish group after 28 days feeding experiment as compared to the reference gene β-actin.

CONCLUSION

In conclusion, the present study has substantiated the various beneficial role of the *A. cepa* as a plausible supplement for fish feed for the use in aquaculture sector. However, further studies are required to validate the studies based on the experimental feeding analysis on aquaculture fields.

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