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Research Article

Dietary supplementation of garlic (*Allium sativum* L.) extract enhances haematological, humoral immune responses and disease resistance of *Mugil cephalus* Linnaeus 1758, larvae against *Photobacterium damselae*

Zorriehzahra M.J.[1]; Adel M.[1*]; Kakoolaki S.[1]; Seidgar M.[2]; Akbari P.[3]; Mehrabi M.R.[1]; Jadgal S.[4]; Sakhaie F.[5]; Fereidouni M.S.[6]

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Abstract

This study was carried out to investigate the effect of dietary supplementation of garlic (*Allium sativum*) extract on growth parameters and hematological parameters and immune system response of *Mugil cephalus* larvae encountered with *Photobacterium damselae*. Two hundred and forty *M. ceph*alus larvae with a mean weight of 5g were randomly divided into 12 equal groups, each containing 3 replicates. Fish were fed with diets containing 50, 100 and 200mg garlic extract/Kg of food for 8 weeks. Based on the results, significant difference was observed comparing final weight, specific growth rate (SGR), daily growth rate (DGR), protein efficiency ratio (PER) and feed conversion ratio (FCR) of 100 and 200mg garlic extract/Kg of food treatments and other treatments (p<0.05). Also, in 100 mg garlic extract/Kg treatment, red and white blood cells, hemoglobin, PCV counts, globulin, total protein and albumin were significantly higher than those of control and 200mg garlic extract/Kg of food (p<0.05). The immune indices (lysozyme activity, serum total immunoglobulin (Ig) content, phagocytic activity and respiratory burst activity) significantly increased in 100mg garlic extract/Kg treatment compared to those of other treatments, especially control (p<0.05). The results revealed that treatments containing 50 and 100mg garlic extract/Kg food had the highest survival after challenging with *P. damselae* compared to survival of other treatments (p<0.05). In conclusion, results suggested that dietary administration of garlic extract; especially in 100mg garlic extract/Kg concentration is recommended for enhancing growth performance, nutritional function, immunity and resistance of *M. cephalus* larvae against the bacterium *P. damselae.*

Keywords: *Allium sativum*, *Mugil cephalus*, Hematological parameters, Immune response, *Photobacterium damselae.*

1-Iranian Fisheries Science Research Institute (IFSRI), Agricultural Research Education and Extension Organization (AREEO), Tehran, Iran.
2-National Artemia Research Center, Iranian Fisheries Science Research Institute, Agricultural Research, Education and Extension Organization, Urmia, Iran.
3-Department of Marine Sciences, Fisheries Group, Chabahar Maritime University, Chabahar, Iran
4-Off-shore Fisheries Research Center, Iranian Fisheries Science Research Institute (IFSRI), Agricultural Research Education and Extension Organization (AREEO), Chabahar, Iran
5-School of Pharmacy, Shahid Beheshti University, Tehran, Iran
6-Animal Health Units, School of Veterinary Medicine, Shiraz University, Shiraz, Iran
*Corresponding author's Email: miladadel85@yahoo.com

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Introduction

M. cephalus is a commercially valuable and popular species that has growing market potential in Iran as well as Europe, East and South Asia (Fereidouni *et al.*, 2015). *M. cephalus* feeds on animal and plant residues (detritus) and plays an important role in the refinement of organic matter resulting from decomposition and maintenance of ecosystem balance.

The innate immune system of fish is improved by many immunostimulant such as levamisole (Ispir and Dörücü, 2005), glucan, and glucan with vitamin C, lipopolysaccharide (Dalmo and Seljelid, 1995) or chitosan (Siwicki *et al.*, 1994). Many plants, such as garlic, are used in treatment and control of diseases of human and animals (Adel *et al.*, 2016). Most of the previous studies focused on the anti-oxidant and anti microbial properties of garlic and its derivatives such as garlic oil and Allison. Most immune stimulants are not used because of high prices and adverse effects, therefore, numerous herbal plants have been examined on aquatic animals (Dugenci *et al.*, 2003). The use of herbal plants as a medicine has been considered in recent years and is used in aquatic animals due to its availability, reasonable price, environmental degradation, lack of environmental impacts and effectiveness against a wide range of pathogens. Many of these plants have immune stimulating properties (Galina *et al.*, 2009).

Garlic (*Allium sativum*) is originated from central Asia. It is one of the main foods of ancient Egyptians which are found in the grave of pharaoh because it was believed to be transmitted to the other world (Harris *et al.*, 2001). Garlic contains more than 200 chemical compounds including sulfur containing volatile oils (allicin, allin, and ajoene) and important enzymes such as myrosinase, peroxidase, allinase. Garlic is properly called one of the wonders of nature, contains important nutritional compounds such as vitamins and minerals, and the antibacterial (Harris *et al.*, 2001), antifungal (Irkin and Korukluoglu 2006), antiparasitic (Fromtling and Bulmer, 1978), antiviral (Corzo-Martinez *et al.*, 2007), immunostimulant (Kyo *et al.*, 1998) and antioxidant (Lee and Gao, 2012) properties.

In previous studies, beneficial effects of *A. sativum* L on growth performance, immunity and disease resistance has been reported in several fish species such as rainbow trout (Fazlolahzadeh *et al.*, 2011), beluga, *Huso huso* (Nobahar *et al.*, 2014), sterlet sturgeon, *Acipenser ruthenus* (Lee *et al.*, 2012), goldfish ,*Carassius auratus* (Dadgar *et al.*, 2019), common carp fingerlings (Karimi Pashaki *et al.*, 2018) and benni fish, *Mesopotamichthys sharpeyi* (Maniat *et al.*, 2014). Also, in the study of Akbari *et al.* (2016) positive effects of *A. sativum* L on feed utilization, carcass composition and survival rate of *M. cephalus* larvae are reported. The aim of this study was to evaluate the effect of dietary supplementation of garlic (*A. sativum* L.) on haematological parameters, humoral immune response and disease resistance of *M. cephalus*

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Linnaeus, 1758 larvae against *Photobacterium damselae.*

Materials and methods

Fish

This study was carried out at the Research Institute of Fisheries, Chabahar (Chabahar, Iran). Two hundred and forty *M. cephalus* larvae with average initial weight of 0.75±0.02g and average length of 4.4±0.81cm were distributed randomly into 4 groups, each one with 60 fish (20 fish per tank) before fed by the experimental diets for 8 weeks. Fish were acclimatized for 2 weeks in plastic tanks (60 L). The health condition of fish was checked visually through their movements, infectious diseases symptoms and physical appearance all over the body and fins of the fish. The water was maintained at 28.2±0.5°C temperature, 7.1±0.87 mg L^{-1} dissolved oxygen and 7.8±0.4 pH during this study. The fish were subjected to a 14L: 10 D photoperiod regime.

Experimental diets and feeding trial

A. sativum dried extract was obtained from the Department of Fisheries, Chabahar Maritime University, Chabahar, Iran. It dried and stored at -20°C until use (Harikrishnan *et al.*, 2003). Components of the basal diet were mixed with *A. sativum* in an appropriate concentration, to get four different experimental diets: 0g (control group), 50, 100 and 200mg/kg of *A. sativum*. The diets were made into pellets, allowed to dry and stored at 4°C until use. During this study, fish were fed (7% of body weight) ten times a day (6:00, 7:30, 9:00, 10:30, 12:00, 13:30, 15:00, 16:30, 18:00, 19:30) for 56 days (Table 1). Daily supplied feed was recorded, and uneaten feed was collected 3h after feeding by syphoning procedure, followed by drying, weighing and finally subtracted from the total amount of supplied diets to calculate the actual feed intake.

All fish were deprived of food 24h before weighing and sampling, and the following parameters were measured at the end of 8 weeks feeding trial (Wahli *et al.*, 2003),

Feed conversion ratio (FCR) = feed intake (g) /weight gain (g).

Protein efficiency ratio (PER) = wet weight gain (g)/ protein intake

Daily Growth Rate (DGR) = $\frac{[(WG\times 100)/(Wi+Wf)/2)]}{t}$.

Where Wi is initial weight, Wf is final weight, WG is weight gain and T is number of days in the feeding period.

Sample collection

At the end of the experimental trial, fish were deprived of food for 24h before sample collection. For blood sample collection, ten *M. cephalus* larvae from each individual tank (30 fish per group) were anaesthetized with clove oil (30mg L^{-1}). Blood samples were obtained from the caudal vein of individual fish (15 fish per treatment, 5 fish per tank). Blood samples were immediately divided into two half parts. One half was transferred to a tube containing anti-coagulant (heparin) for studying the respiratory burst assay and make hematological

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analysis, while the other half was transferred to non-heparinized tubes for biochemical and immunological studies. Sera samples were isolated by blood centrifugation Hettich model DV200 (3000 rpm, 10 min) and stored at -20ºC until use.

Table 1: Dietary formulation and proximate composition of the basal diet.

Ingredient	Composition (%)
Fish meal	48.0
Wheat flour	18.7
Soy bean meal	21.0
Soy bean oil	3.0
Fish oil	3.0
Mineral premix[a]	1.5
Vitamin premix[a]	2.0
Binder[b]	2.0
Antifungi[c]	0.4
Antioxidant[d]	0.4
Total	100
Proximate composition (% dry weight)	
Crude protein	50.6
Crude lipid	12.9
Ash	14.8
Fiber	1.5
Moisture	6.3
NFE[e]	15.6

[a]Premix detailed by Fereidouni et al. (2015).
[b]Ametbinder™, MehrTaban-e-Yazd, Iran.
[c]ToxiBan antifungal (Vet-A-Mix, Shenan- doah, IA).
[d]Butylated hydroxytoluene (BHT, Merck, Germany).
[e] Nitrogen-free extracts (NFE) = dry matter - (crude protein + crude lipid + ash + fiber).

Hematological assays

To measure blood parameters, conventional methods of mammalian blood parameters were used with little change (Rehulka, 2000). Total red blood cells (RBC: 10^6 mm^{-3}) and white blood cells (WBC: 10^3 mm^{-3}) were enumerated in an improved Neubaeur hemocytometer using Hayem and Turck diluting fluids (Rehulka, 2000). Hematocrit (HCT, %) was determined by standard microhematocrit method and expressed as percentage (Rehulka, 2000). Hemoglobin (Hb, g dl^{-1}) level was determined according to cyanomethemoglobin procedure according to Trenzado *et al.* (2009).

Biochemical analysis

Globulin, total protein and albumin were estimated at 35ºC in fish sera using commercial kits (Pars Azmoon Company, Tehran, Iran) and a biochemical auto analyzer (Eurolyser, Belgium).

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Immune parameters

Serum lysozyme activity was measured according to the method described by Ellis (1999) with some modifications. Briefly, 25µL of sera was added to 1mL of a suspension of *Micrococcus lysodeikticus* (0.2 mg mL^{-1}) in a 0.05M sodium phosphate buffer (pH 6.2) and the absorbance was measured at 670nm after 30s and 180s by spectrophotometer (Biophotometer, Eppendorf).

Phagocytic activity of blood leukocytes was measured using May–Grunwald–Giemsa-stained yeast cells (Baba *et al.*, 2015) that had been phagocytized by the cells. One hundred microliters of the leukocyte solution were mixed with 2000µL of boiled yeast solution (preparing a yeast cell-to-leukocyte ratio of 20:1). The combination was then incubated at laboratory temperature for 60 min by gentle shaking. Following incubation, this mixture was smeared on the glass slides and allowed slides to be air-dried, fixed in 95% ethanol, re-dried and stained with May–Grunwald–Giemsa. The phagocytic cells and phagocytosed yeast were counted, and PA was determined by evaluating 100 phagocytes using a light microscope (Baba *et al.*, 2015).

PA = (number of phagocytic cells with engulfed yeast/number of phagocytic cells) × 100:

Serum total immunoglobulin (Ig) content was measured according to the method described by Adel *et al.* (2015) using a microprotein determination method (C-690; Sigma), prior and after precipitating down the immunoglobulin molecules by means of a 12% solution of polyethene glycol (Sigma). The difference in protein content was considered as the IgM content.

Finally, respiratory burst activity in leucocytes was measured by chemiluminescent assay (CL) by a method described by Secombes and Chung (1988) using CL analysis (LUMI scan Ascent T392, Finland). Light emission results are expressed in the form of relative light units per second (RLU s^{1}) recorded by luminometer.

Challenge test

P. damselae (SK7 strain) which had been originally isolated from suspected fish were obtained from Iran Veterinary Organization (IVO, Chabahar, Iran) and cultured in tryptic soy broth (TSB, Merck, Darmstadt, Germany) at 25°C for 48h. After 56 days week-feeding with different doses of garlic extract and control groups (10×3=30 fish per group) were challenged by immersion (Im) of *P. damselae* in 0.9% (w/v) saline containing 7.2×10^{4} cells mL^{-1} (Akbari and Kakoolaki, 2019). All fish were fed with the basal diet thereafter and kept under observation for 10 days to record any abnormal behavior, clinical signs and mortality. At the end of the challenge test, cumulative survival rate (%) was calculated.

Statistical analysis

All the tests were performed in triplicate. The data were subjected to statistical analysis using the SPSS software version no. 21 (SPSS Inc., Chicago, IL, USA). The statistical analysis was done

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by using one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. *P*-value of <0.05 was considered significant.

Results

Growth indices

The results of growth and nutrition indices are shown in Table 2. Based on these results, significant difference was observed in final weight, specific growth rate (SGR), daily growth rate (DGR), protein efficiency ratio (PER) and feed conversion ratio (FCR) in 100 and 200mg garlic extract/Kg of food treatments compared to other treatments (p<0.05). Highest rates of mentioned parameters were observed in 100mg/kg garlic extract treatment (p<0.05). Also final weight, daily growth rate, protein efficiency ratio and feed conversion ratio of 50mg of garlic extract per kg of food did not show significant difference with control treatment (p>0.05).

Table 2: Growth performance of *M. cephalus* larvae fed diets enriched with different levels of garlic extract (0, 50, 100 and 200mg/kg) for 8 weeks.

Parameter	Treatments (mg/kg of garlic extract)			
	0	**50**	**100**	**200**
Initial weight (g)	0.75±0.06[a]	0.75±0.03[a]	0.74±0.03[a]	0.68±0.02[a]
Final weight (g)	4.59± 0.17 [b]	4.68± 0.28 [b]	5.02± 0.16 [a]	5.20± 0.29 [a]
DGC	0.88± 0.02 [b]	0.89± 0.01 [b]	1.05± 0.01 [a]	1.07± 0.04 [a]
FCR	1.14±0.12[a]	1.10±0.10[a]	0.92±0.20[b]	0.93±0.31[b]
PER	1.50± 0.10 [c]	1.85± 0.13 [b]	1.98± 0.21 [a]	2.01± 0.13 [a]

Data are presented as mean ± S.D. Values in each row with different superscripts show significant difference (p< 0.05).

Hematological indices

The range of RBC, WBC, Hemoglobin (Hb), Hematocrit (HCT) changes in different treatments are shown in Figure 1. The number of WBC and RBC counts from control to 100mg/kg garlic extract treatment increased gradually and decreased in 200mg/kg level. Hemoglobin levels were significantly different in studied treatments (p<0.05). Lowest and highest amounts of hemoglobin were observed in the treatment containing 200mg/kg garlic extract and in the treatment containing 100mg/kg garlic extract, respectively. Changes in hematocrit levels in different treatments did not show any particular trend. Lowest and highest levels of hematocrit were observed in control and 50mg/kg garlic extract treatments.

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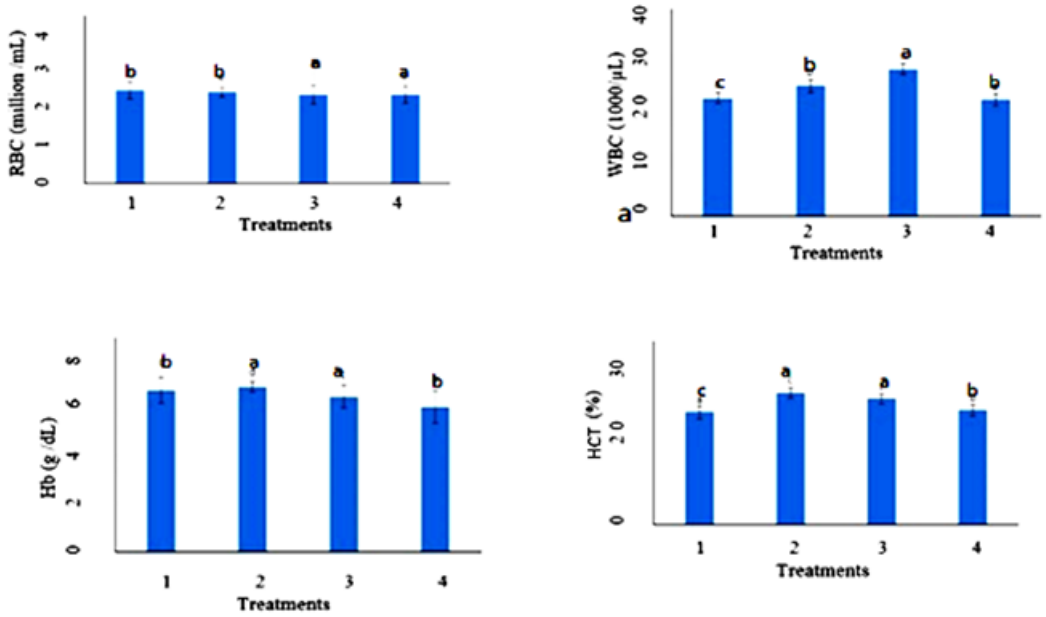


Figure 1: Hematological profile of *M. cephalus* larvae fed for 8 weeks with diets enriched with different levels of garlic extract (Treatments 1 to 4 contain 0, 50, 100 and 200mg/kg garlic extract, respectively). Data are presented as mean±S.D. Values with different superscripts show significant difference (p<0.05).

Biochemical parameters

Effects of garlic extract on total proteins, albumin, and globulin levels of grey mullet serum are shown in Figure 2. The levels of globulin, total proteins and albumin in the serum of fish fed with diets enriched with different levels of garlic extract were significantly increased compared to those of the control (p<0.05). Also the highest levels of globulin, total protein and albumin were observed in the treatment containing 100mg garlic extract/kg of food (p<0.05).

Immune parameters

Mean changes in lysozyme activity, serum total immunoglobulin (Ig) content, phagocytic activity and respiratory burst activity are shown in Figure 3. The immune indices (lysozyme activity, serum total immunoglobulin (Ig) content, phagocytic activity and respiratory burst activity) increased significantly in 100mg garlic extract/kg of food treatment compared to other treatments, especially the control (p<0.05). Although, no significant difference was observed between the treatment containing 50mg of garlic extract per kg of food with 200mg garlic extract/kg of food (p<0.05).

Cumulative mortality rate after challenge with Photobacterium damselae bacteria

The percentage of cumulative mortality after challenge with *P. damselae* for 10 days in various experimental treatments is shown in Figure 4. Results revealed that treatment containing 100mg garlic extract/Kg food had the highest survival after challenging with *P. damselae* compared to other treatments (p<0.05).

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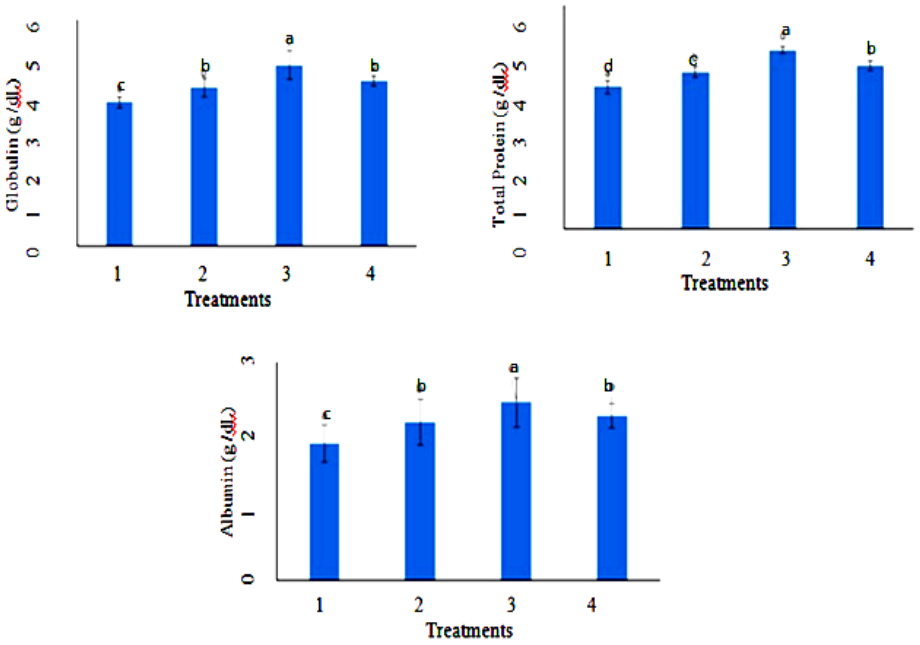


Figure 2: Biochemical profile of *M. cephalus* larvae fed for 8 weeks with diets enriched with different levels of garlic extract (Treatments 1 to 4 contain 0, 50, 100 and 200mg/kg garlic extract, respectively). Data are presented as mean±S.D. Values with different superscripts show significant difference (p<0.05).

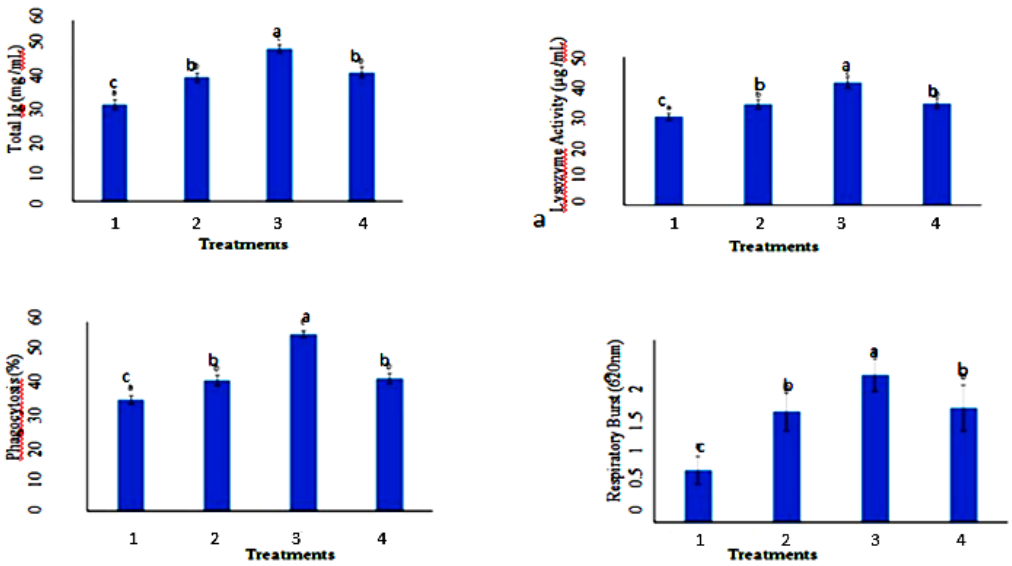


Figure 3: lysozyme activity, serum total immunoglobulin (Ig) content, phagocytic activity and respiratory burst activity of *M. cephalus* larvae fed for 8 weeks with diets enriched with different levels of garlic extract (Treatments 1 to 4 contains 0, 50, 100 and 200mg/kg garlic extract, respectively). Data are presented as mean±S.D. Values with different superscripts show significant difference (p<0.05).

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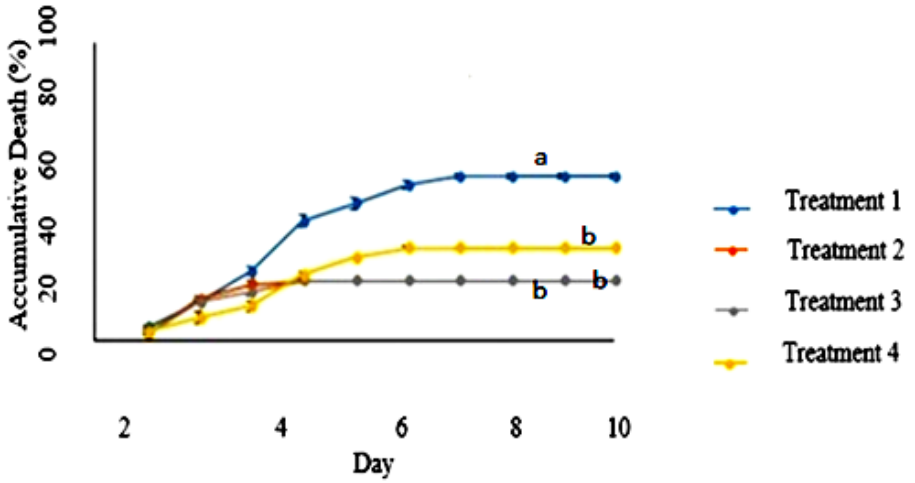


Figure 4: The percentage of cumulative mortality in *M. cephalus* larvae fed with diets supplemented with garlic extract against *Photobacterium damselae* (Treatments 1 to 4 contains 0, 50, 100 and 200mg/kg garlic extract, respectively). Data are presented as mean±S.D. Values with different superscripts show significant difference (p<0.05).

Discussion

Based on the results, significant difference was observed in final weight, specific growth rate (SGR), daily growth rate (DGR), protein efficiency ratio (PER) and feed conversion ratio (FCR) in 100 and 200mg garlic extract/Kg treatments compared to other treatments (p<0.05). In a similar study, highest weight gain, the best percentage of protein efficacy ratio, the highest protein production rate and the lowest food intake were observed in 150mg/kg garlic essential oils compared to control (Khalil *et al.*, 2001). Khalil *et al.* (2001) confirmed that allicin in garlic plant enhance the intestinal function, improved nutrition and better utilizes energy and finally improved the growth performance. Lee *et al.* (2012) showed that a diet containing 0.5% of garlic extract improve the growth performance of *Acipenser ruthenus*. Garlic extract (30 g/kg) in Nile tilapia (*O. niloticus*) led to better weight gain, protein efficiency ratio and decreased feed conversion ratio (Shalaby *et al.*, 2006). Contrary to these results, there was no significant difference in growth performance of African catfish (*Clarias gariepinus*) that received garlic extracts (Nwabueze, 2012). These differences may be related to age or size of fish, fish species, culture conditions, diet composition, level of garlic extract and different kinds of garlic plant (powders, oils or extracts) used in the feed (Akrami *et al.*, 2015).

The number of WBC and RBC counts increased gradually from control to 100mg/kg garlic extract treatment and then decreased in 200mg/kg level. In a similar study, Shalaby *et al.* (2006) showed that increased levels of garlic in tilapia fish diet increased the RBC count. The haemoglobin (Hb) in the blood has an important role and acts as carrier for oxygen in tissues. Hemoglobin levels were significantly different in the

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studied treatments (p<0.05). Lowest and highest amount of hemoglobin were observed in the treatment containing 200mg/kg garlic extract and in the treatment containing 100mg/kg garlic extract, respectively. In a similar study, Chitsaz *et al.* (2018) showed that, haemoglobin content was significantly higher in 1% garlic peel than other studied diets (0. 0.5%, 1.5% and 2%). Changes in hematocrit levels in different treatments did not show any particular trend. Lowest and highest levels of hematocrit were observed in the control and 50mg/kg garlic extract treatments. Nwabueze (2012) reported that addition garlic extract to *Clarias garepinus* diet, significantly increased HCt, RBC and Hb indices in 0.5% garlic group compared to other treatments. Also, in Saleh *et al.* (2015) study, significant increase in hemoglobin content and hematocrit value were observed in sea bass (*Dicentrarcus labrax*) fry feed with garlic powder compared to the control group.

Albumin and globulin levels in serum are dependent on the amount of total protein in the blood serum (Akrami *et al.*, 2015). Albumin is synthesized in the liver, and it is important to maintain osmotic pressure and also act as a carrier of various substances, including lipids, hormones, minerals and vitamins, and plays an important role in the transport of compounds such as medications in the blood and can transport the compounds such as drug in the blood (Adel *et al.*, 2015). The levels of globulin, total proteins and albumin in serum of fish fed with diets enriched with different levels of garlic extract increased significantly compared to control (p<0.05). Also the highest levels of globulin, total protein and albumin were observed in the treatment containing 100mg garlic extract/kg of food (p<0.05). Shalaby *et al.* (2006) reported that factors such as sex, spawning, food, osmotic pressure, temperature, light, age, and oxygen can affected serum total protein. They also showed that the use of 20 and 30g of garlic per kg of diet resulted in a significant increase in the plasma protein content of Nile tilapia (*O. niloticus*) compared to the control. Chitsaz *et al.* (2018) also showed that the use of garlic's essential oil resulted in a significant increase in plasma protein concentrations in beluga juvenile (*H. huso*) compared to control treatment. Sahu *et al.* (2006) reported that addition of 0.1, 0.5 and 1% of garlic to the diet resulted in a significant increase in plasma protein and globulin level compared to control treatment in *L. rohita*.

The results of this study showed that the immune indices including lysozyme activity, serum total immunoglobulin (Ig) content, phagocytic activity and respiratory burst activity significantly increased in the 100mg garlic extract/kg of food treatment compared to other treatments, especially control (p<0.05). *Allium* species play an important role in improving the immune system by increasing immune responses such as increasing lymphocyte synthesis, increasing cytokine release, and

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increasing phagocytic activity. Sahu *et al.* (2006) reported that the use of garlic extract with doses of 1, 5 and 10g per kg of food resulted in a significant increase in anion superoxide, lysozyme activity, total protein, albumin, and bactericidal activity of *Labeo rohita* compared with the control. Also, Ndong and Fall (2011) showed that addition of 0.5 grams per kilogram of garlic in hybrid tilapia (*O. niloticus* × *O. aureus*) feed significantly increased the number of leukocytes, respiratory burst activity, phagocytic and lysozyme activities.

Total immunoglobulins (Ig) play an important role on the immunity of fish (Sun *et al.*, 2012). Several studies have shown that total immunoglobulin levels are associated with factors such as age, environmental conditions, fish health status, the use of different immune stimulants, and concentration and time of exposure (Picchitti *et al.*, 2001). The results of this study showed that the addition of 100mg garlic extract/kg of diet showed the highest plasma Ig level compared to the other treatments. In Karimi Pashaki *et al.* (2018) study, highest levels of lysozyme and IgM were reported in common carp fingerlings fed with a diet containing 5g kg^{-1} of garlic extract. Talpur and Ikhwanuddin (2012) reported that using 10g kg^{-1} garlic in the *Lates calcarifer* diet had significant increase in lysozyme activity and IgM level. The effects of immune stimulation and increase of fish resistance following consumption of garlic have been related to the presence of allicin, vitamin A and vitamin C in garlic plant (Khodadadi and Nosrati, 2012).

Photobacteriosis caused by bacterial strain of *P. damselae* is a major problem in aquaculture industry, which leads to high mortality rates in marine fish. This negative gram bacterium was often observed in Japanese sea bass and Japanese yellow tail (Bakopoulos *et al.*, 1997). The present study showed that the fish fed with 100mg garlic extract/kg of food had the lowest cumulative mortality compared to the other garlic extract treatments and control group after challenge with *P. damselae* (p<0.05). The highest cumulative mortality (55%) was observed in control treatment (p<0.05). Our results are in agreement with those obtained on *M. cephalus* fed with *Echinacea purpurea*-supplemented diet against *P. damselae* infections. Also, mortalities of Nile tilapia (*Oreochromis niloticus*) following challenge with *Pseudomonas fluorescens* were lower in groups that received garlic compared to the control treatment (Diab *et al.*, 2008). In a similar study, Adel *et al.* (2016) showed that resistance of rainbow trout fed with diets containing 2% and 3% *Mentha piperita* plant extract were higher than those of the control group after challenging with *Y. ruckeri*.

In conclusion, the results suggested that dietary administration of garlic extract especially in the 100mg garlic extract/Kg concentration is recommended for enhancing growth performance, nutritional function, immunity and resistance of *M. ceph*alus larvae against *P. damselae.*

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