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Some Biochemical and Immunological Changes Associated with Use of Garlic Extract (allicin) in Combating Some Fish Pathogens

Riad Khalil1, Hana El-Hofy[2], Manal M.Yehya[2] Khalid M. Selim[4]

[1]Department of Fish and Poultry diseases, Faculty Veterinary Medicine Alexandria University, [2]Animal Health Research Institute Damanhour Branch, [4] Department of Fish Diseases and Management Faculty Veterinary Medicine Zagazig University.

Key words
allicin, sea bream, bactericidal activity, Cortisol, histopathology.

ABSTRACT:

We studied the effects of garlic (allicin) which used as immunostimulant on some physiological, biochemical parameters, survival rate, histopathological studies and bacteriological characteristics of sea bream. Fish (50 ± 5 g/fish) were assigned to 3 treatments, with three replicates each. Treatment groups had a different level of allicin (Garlen®), (1 and 2 gm. /kg feed) added to their diets; the control group diet was free from garlic. Diets also contained 25% crude protein (CP) and were administered at a rate of 3% live body weight twice daily for 8 weeks. At the end of 7th week ten fish from each treatment group and from the control were artificially infected by intra peritoneal injection with 0.2 ml of culture suspension of pathogenic *Vibrio alginolyticus* previously adjusted to 10^4.Results showed that the differential leucocytic count, phagocytic activity, phagocytic index, serum lysozomal and bactericidal activity increased significantly with increasing levels of allicin. Plasma Cortisol level decreased significantly with increasing levels of allicin. Total protein and globulin were also increased significantly in the treated groups than the control group. All bacterial counts decreased significantly in treated groups than the control in all weeks. Treated groups had lower mortality rate than the control group during the challenge test. Histopathological studies of control group revealed congestion in blood vessel of gills, liver, spleen and kidney with, hyperplasia of epithelial cells between secondary lamellae led to fusion, edema and lifting of the lamellar epithelium of secondary lamellae in gills, vacuolar degeneration of hepatocytes, with infiltration by monocytes in liver, dilation of blood vessel, severe depletion of white bulb ,edema, and hyperactivation of melanomacrophage center in spleen ,necrosis of renal tubules epithelium, and inter renal haemopoietic tissues with atrophied glomerular tuft and widening of bowman capsules in kidney. Moderate to minimal histopathological changes appeared in all organs in groups received (Garlen®) according to its dose.

Corresponding Author: *Ryad, Khalil*: e-mail: ryadvet_2013@yahoo.com

1. INTRODUCTION:

The use of the immunostimulants in aquaculture is becoming popular, enhancing the activity of the non-specific defense mechanisms and increasing disease resistance**,** mainly through facilitate the function of phagocytic cells, increase their bactericidal activities, and stimulate the natural killer cells, complement system, lysozyme activity, and antibody responses in fish. (Dalmo and Seljelid, 1995, Raa, 1996 and Harikrishnan et al., 2011). Garlic can help in the control of pathogens, especially bacteria and fungi, and increase the welfare of fish (Adetumbi et al., 1986; Ress et al., 1993 and Corzo-Martinez et al., 2007). Garlic, Allium sativum , has been used for the treatment of many diseases since ancient times as reported in the Codex Ebers (1550 BC), where an Egyptian medical papyrus described several therapeutic formulas based on the garlic as a useful remedy for a variety of diseases such as heart problems, headache, bites, worms and tumors (Block, 1985). Cloves of garlic were found in the tomb of Tutankhamen and in the sacred underground temple of the bulls of Saqqara. It has long been considered that garlic (Allium sativum) has several beneficial effects for

human and animals, exhibiting antimicrobial, antioxidant, and antihypertensive properties (Konjufca et al., 1997 and Sivam, 2001). Previous research suggested that those functions are mainly attributed to the bioactive components of garlic, including sulphur containing compounds, such as allicin, diallylsulphides and allicin (Amagase et al., 1993). Many beneficial health properties of garlic are attributed to organosulphur compounds, particularly to thiosulfinates (Block, 1992). Allicin (diallythiosulfinate) is the most abundant compound representing about 70% of all thiosulfinates present, or formed in crushed garlic (Block, 1992 and Han et al., 1995). Garlic has proven to be hypolipidemic (Sumiyoshi, 1997), antimicrobial (Kumar and Berwal, 1998), antihypertensive (Suetsuna, 1998), hepatoprotective and insecticidal (Wang et al., 1998). Garlic extract has also been shown to reduce serum cholesterol levels and increase blood coagulation time (Bordia et al., 1975 and Augusti, 1977)). An antifungal activity has been identified in garlic bulbs (Fromthing and Bulmer, 1978). S-allyl cysteine, present in the crushed garlic, was found to inhibit tumor metabolism and enhance the immune response (Sumiyoshi, 1997). The allyl sulfides enhance the glutathione S-transferase enzyme systems, which through their dependent biochemical pathways enhance the liver's detoxification of carcinogenic substances. The allium species show immune enhancing activities that include promotion of lymphocyte synthesis, cytokine release, phagocytosis and natural killer cell activity (Kyo et al, 1998).

The present study was conducted to evaluate the efficiency of the garlic (Allicin) in improving the immune response, survival, physiological, biochemical parameters, histopathological studies and disease resistance in sea bream.

2. MATERIALS AND METHODS

I- Materials:

1. Fish:

A total number of 300 apparently healthy ***sea bream,*** with average body weight of (50±5g/fish) were obtained from Private marine fish farm at Borg AL Arab at Alexandria Governorate. Fish were transported a live to the laboratory of the same farm.

2. Aquaria:

Fish were kept in prepared glass aquaria (90×50×35 cm). These aquaria were used for holding the experimental fish throughout the period of the present study, (triplicate each treatment), supplied with marine water according to (Innes, 1966).

3. Yeast strain:

The *Candida albicans* strain was kindly supplied by the department of poultry and fish diseases Fac. Vet. Med., Alexandria University which is used for phagocytic assay study.

4. Bacterial strain:

Bacterial strain *Vibrio alginolyticus* strain was kindly supplied by the department of poultry and fish diseases Fac. Vet. Med., Alexandria University which is used for the serum bactericidal activity study, bacterin preparation and challenge test.

Bacterial strain lyophilized *Micrococcus lysodekticus* which is used for serum lysozomal activity (Sigma M 3770).

5. Media used:

5. a. Media used for *Candida albicans* culture was:

-Sabaroud, s dextrose broth (Oxoid, 1982).

5. b. Media used for bacterial growth:

-Trypticase soya agar (TSA), BBL, Cat. No. 11764.

-MacConkey broth and agar media which is used for isolation and propagation of the number of Micrococcus lysodekticus (Oxoid, 1982).

6-Kits for clinico- biochemical analysis:

Kits for total protein, serum albumin and cortisol (Pasteur, Lab, France).

7- Commercial product used:

Garlen immunostimulants**:** which consists of garlic extract (allicin 25%)?

II- Methods

1- The experimental design:

The experiment aimed to evaluate the effects of commercial immunostimulants (Garlen®) on the immune response and the health conditions of *sea bream.* The experiment was carried out from September 2012 till the end of November 2012. The experimental fish, seabream were obtained from Private marine fish farm at Borg AL Arab at Alexandria Governorate. All fish were apparently healthy from any pathogenic bacteria and free from parasitic infestation and equated with similar size (averaging 50 ± 5 gm. in weight).The water quality parameters contained the following concentrations: 144.5 ± 8 mg $CaCo_3$ /l total hardness, 8.2± 0.3 ppm dissolved oxygen, 0.01mg/ l Nitrite (No2), 0.02 mg /l Nitrate (No_3), 20± 7 mg/ l H_2s and 0.01 mg/l un ionized ammonia. The 1st group used as control and fed on basal diet only without any treatment. The 2nd group fed on basal diet and 1 gm. /kg feed (Garlen®) immunostimulant. The 3rd group fed on basal diet and 2 gm. / kg feed (Garlen®) immune stimulant. . Two thirds of water was changed day after day.

2- Blood sampling:

At zero days, 2nd, 4th, 6th, 8th weeks during the experimental period, 2 ml blood samples were collected from different groups via the caudal vessels from 2 fish using disposable syringe (Hawak et al., 1965). Phagocytic assay according to Kawahara et al. (1991). The collected serum was used for biochemical determination (Lied et al., 1975).

3-Differential leucocytic count:

Blood film was taken and prepared according the method described by ***Lucky (***1977). The percentage and absolute value for each type of cells were calculated according to Schalm (1986).

4- Determination of phagocytic activity and phagocytic index:

Phagocytic activity was determined according to kawahara et al. (1991). Results were expressed as means ± S.E. and differences were evaluated by Student's t-test.

Phagocytic activity (PA) = percentage of phagocytic cells containing yeast cells.

$$\text{Phagocytic index (PI)} = \frac{\text{Number of yeast cells phagocytized}}{\text{Number of phagocytic cells}}$$

5-Clinico-biochemical analysis:

5.1. Determination of serum total protein:

Serum total protein was determined according to Doumas et al. (1981) using commercial kits produced by Pasteur Lab.

5.2. Determination of serum albumin:

Serum albumin was determined according to Reinhold (1953) using commercially available kits of Chemroy.

5.3. Determination of serum globulin:

Serum globulin was determined by subtract the total serum albumin from total serum protein according to (Coles, 1974 and Khalil, 2000).

5.4 Determination of serum albumin/globulin ratio:

Determined by division of serum albumin value on serum globulin value.

5.5 Determination of cortisol:

Radioimmunoassay of cortisone in serum (Gilles et al., 1997).

6- Determination of serum bactericidal activity:

Serum bactericidal activity to *Aeromonas hydrophila* strain was determined according to *Rainger and Rowley (1993).* The results were recorded as survival index (SI) (Wordlow and Unlles, 1978). Values were calculated as follows:

SI= CFU at end / CFU at start × 100.

7- Determination of serum lysozyme activity:

Serum lysozyme activity was measured with the turbidimetric method described by Engstad et al.(1992).The results was expressed as one unit of lysozyme activity was defined

as a reduction in absorbency of 0.001/min.

Lysozyme activity = (A0- A) / A.

8-Determination of total bacterial, total enterobacteriaceae and total coliform counts (APHA, 1992):

One gram of mucous was collected from the all treated groups. The all plates incubated at 28 C° for 24-48hrs then counted of the all growth colonies (APHA, 1992**).**

9- Antibody titration against *Vibrio alginolyticus*:

Detection of immune response to *Vibrio alginolyticus* was evaluated by microagglutination (MA) test according to the method described by Badran (1990) and Khalil (2000). Agglutination titers were expressed as log 2 of the highest serum dilution still giving a clear agglutination (Badran, 1990).

10- Challenge test:

At the end of 7th week ten fish from each treatment group and from the control were clinically examined and blood samples bacteriologically tested and determined to be free from bacterial infection, were then artificially infected by intra peritoneal injection with 0.2 ml of culture suspension of pathogenic *Vibrio alginolyticus* previously adjusted to 10^4 specificity of death was determined by re isolation of injected bacteria from freshly dead fish during the period of observation. (One week) according to Soliman (1988 a). The relative level of protection (RLP), among the challenged fish was determined according to Ruangroupan et al., (1986) using the following equation.

$$RLP = 100 - \frac{\text{Percentage of immunized mortality}}{\text{Percentage of control mortality}} \times 100$$

$$\text{Mortality \%} = \frac{\text{NO of death in a specified period}}{\text{Total population during that period}}$$

11-Histopathological studies:

Specimens were freshly taken from different organs of fish (Gills, liver, spleen, and kidney), specimens were trimmed and fixed in 10% neutral formalin for about 24 hours then rinsed and dehydrated with a graded ethanol series, and cleared in xylol then embedded in paraffin wax and sectioned to 5 µ thickness, stained with Harris Hematoxyline and Eosin and examined microscopically according to Sonia et al. (2007).

12-Statistical analysis:

The data of hematological and biochemical examinations of exposed fish were statistically analyzed using t-test, Duncan-test after ANOVA and simple correlation according to (SAS, 1987) to examine the significant effect of the main variables on the studied parameters. After that the results presented in the form of figures according to Harvard graphics (HGW-4) computer program.

3. RESULTS

1-Effects of different treatments of Garlen® on differential leucocytic count in *O. niloticus* blood:

Table (1) explained the significant ($P<0.05$) effect of different treatments among different weeks on differential leucocytic count in *sea bream* blood. In zero days (at the beginning of the experiment) results of differential leucocytic count revealed no significant value in all groups.

Table 1. Differential leucocytic count among different groups in different weeks.

	Groups	Lymphocytes	Monocytes	Basophils	Eosinophil	Neutrophils	Thrombocytes
		Mean ± Std .Error	Mean ± Std. Error	Mean ± Std. Error	Mean ± Std. Error	Mean ± Std. Error	Mean ± Std. Error
Zero day	Control*	60.67±0.88[a]	1.67±0.33[a]	5.00±0.58[a]	8.33±0.33[a]	21.00±1.15[a]	3.33±0.33[a]
	Garlen 1 gm./kg feed	61.00±0.58[a]	2.33±0.33[a]	5.67±0.67[a]	7.00±0.58[a]	19.67±1.45[a]	3.35±0.33[a]
	Garlen 2 gm./kg feed	59.97±2.03[a]	2.33±0.33[a]	4.67±0.33[a]	7.67±0.33[a]	22.00±2.08[a]	3.36±0.58[a]
2nd week	Control	59.33±1.20[c]	1.67±0.33 [a]	6.00±0.58 [a]	8.00±0.58 [a]	20.33±1.76 [a]	2.67±0.33 [a]
	Garlen 1 gm./kg feed	64.67±0.88[ab]	1.67±0.33 [a]	6.63±0.00 [a]	8.67±0.33 [a]	13.67±1.20 [b]	3.33±0.33 [a]
	Garlen 2 gm./kg feed	66.00±0.58 [a]	1.67±0.33 [a]	5.20±1.20 [a]	9.00±0.00 [a]	14.45±1.76 [b]	3.68±0.58 [a]
4th	Control	59.00±0.58[c]	1.33±0.33[a]	6.00±0.58[a]	6.67±0.67[a]	25.33±0.33[a]	2.33±0.33[a]
	Garlen 1 gm./kg feed	67.00±0.58[b]	2.00±0.58[a]	5.67±0.33[a]	7.00±0.58[a]	14.67±1.33[b]	3.67±0.33[a]
	Garlen 2 gm./kg feed	70.00±0.58[a]	1.33±0.33[a]	5.67±0.33[a]	6.67±0.33[a]	12.67±1.33[c]	3.67±0.67[a]
6th	Control	56.67±0.88[c]	1.67±0.33[a]	6.33±0.33[a]	8.33±0.33[a]	23.33±0.67[a]	3.00±0.58[a]
	Garlen 1 gm./kg feed	68.67±0.33[b]	2.00±0.58[a]	7.67±0.33[a]	7.67±0.33[a]	10.67±1.76[b]	3.33±0.33[a]
	Garlen 2 gm./kg feed	73.33±0.88[a]	1.67±0.33[a]	7.33±0.67[a]	7.33±0.88[a]	9.67±2.91[c]	4.00±0.00[a]
8th	Control	59.00±0.58[c]	2.33±0.33[b]	6.33±0.33[a]	7.00±0.58[a]	22.00±2.08[a]	3.33±0.33[a]
	Garlen 1 gm./kg feed	71.67±0.88[b]	3.67±0.33[a]	6.33±0.88[a]	8.00±0.58[a]	6.33±1.45[b]	4.00±0.58[a]
	Garlen 2 gm./kg feed	75.00±0.58[a]	3.67±0.33[a]	6.33±0.88[a]	6.67±0.33[a]	4.00±1.53[c]	4.33±0.33[a]

For each week: Means within the same column of different letters are significant different at (P<0.05).
*Number of fish = 3

Table 2. Phagocytic activity and phagocytic index among different groups in different weeks.

	Groups *	Phagocytic activity	Phagocytic index
		Mean ± Std. Error	Mean ± Std. Error
Zero day	Control	21.33±0.88[a]	2.17±0.03[a]
	Garlen 1 gm./kg feed	20.00±0.58[a]	2.20±0.06[a]
	Garlen 2 gm./kg feed	20.33±0.33[a]	2.23±0.07[a]
2 nd week	Control	20.33±0.33[c]	2.17±0.03[c]
	Garlen 1 gm./kg feed	23.67±0.33[b]	2.87±0.12[b]
	Garlen 2 gm./kg feed	24.33±0.33[a]	3.13±0.12[a]
4th week	Control	20.33±0.33[c]	2.17±0.07[c]
	Garlen 1 gm./kg feed	25.33±0.33[b]	2.77±0.03[b]
	Garlen 2 gm./kg feed	27.67±0.33[a]	2.93±0.03[a]
6th+ week	Control	20.33±0.33[c]	2.23±0.09[c]
	Garlen 1 gm./kg feed	25.67±0.33[b]	2.93±0.09[b]
	Garlen 2gm/kg feed	28.33±0.33[a]	3.23±0.03[a]
8h week	Control	21.00±0.58[c]	2.27±0.09[c]
	Garlen 1 gm./kg feed	27.67±0.33[b]	2.83±0.03[b]
	Garlen 2 gm./kg feed	30.33±0.33[a]	3.37±0.03[a]

For each week: Means within the same column of different letters are significantly different at P<0.05).
*Number of fish = 3

2- Effects of different treatments of Garlen® on phagocytic activity and phagocytic index in sea bream blood:

We can notice that there was progressive increasing in phagocytic activity and phagocytic index in the groups treated with Garlen® immunostimulant (high dose then small dose), than the control group from the 2nd week till 8th week as indicated in Table (2).

3- Effects of different treatments of Garlen® on serum lysozyme and bactericidal activity:

The serum lysozyme and bactericidal activity were significantly elevated progressively in the groups treated with Garlen® immunostimulant (high dose then small dose), than the groups treated with probiotics and also than the control group from the 2nd week till 8th week as indicated in Table (3).

4-Effects of different treatments of Garlen® on cortisol level of sea bream:

Cortisol hormone levels were significantly decreased progressively in the groups treated with Garlen® immune stimulant (high dose then small dose) than the control group in zero day (at the beginning of the experiment) and in 2nd, 4th, 6th, 8th weeks as shown in Table (4).

Table 3. Serum lysozyme and bactericidal activity among different groups in different weeks.

	Groups	Lysozome activity	Bactericidal activity
		Mean ± Std. Error	Mean ± Std. Error
Zero day	Control*	0.02±0.00[a]	4.30±0.00[a]
	Garlen 1gm/kg feed	0.03±0.00[a]	3.70±0.00[a]
	Garlen 2 gm./kg feed	0.03±0.00[a]	3.80±0.00[a]
2nd week	Control	0.02±0.00[c]	3.93±0.09[c]
	Garlen 1 gm./kg feed	0.06±0.00[b]	5.27±0.20[b]
	Garlen 2 gm./kg feed	0.08±0.01[a]	5.63±0.38[a]
4th week	Control	0.02±0.00[c]	3.87±0.12[c]
	Garlen 1 gm./kg feed	0.10±0.01[b]	5.73±0.09[b]
	Garlen 2 gm./kg feed	0.16±0.01[a]	6.33±0.12[a]
6th week	Control	0.01±0.00[c]	3.67±0.09[c]
	Garlen 1 gm./kg feed	0.11±0.01[b]	6.20±0.10[b]
	Garlen 2 gm./kg feed	0.19±0.02[a]	7.00±0.15[a]
8th week	Control	0.01±0.00[c]	3.70±0.15[c]
	Garlen 1 gm./kg feed	0.21±0.03[b]	5.77±0.24[b]
	Garlen 2 gm./kg feed	0.32±0.03[a]	8.63±0.34[a]

*Number of fish = 3, For each week: Means within the same column of different letters are significantly different at (P<0.05).

5- Effects of different treatments of Garlen® on (Total proteins, albumin, globulin and albumin/globulin ratio):

In zero days (at the beginning of the experiment) results showed no significant value in total proteins, albumin, and globulin and albumin/globulin ratio in all groups of sea bream. Otherwise the serum total proteins, and globulin were significantly elevated progressively in the groups treated with Garlen® immunostimulant (high dose then small dose), than the control group from the 2nd week till 8th week. Serum albumin and albumin/globulin ratio were significantly decreased in the groups treated with Garlen® immunostimulant (both doses) than the control group in all weeks as shown in Table (5).

Table 4. Cortisol level, among different groups in different weeks

	Groups	Cortisol level (P/mol L)
		Mean ± Std. Error
Zero day	Control	547.98±2.85[a]
	Garlen 1 gm./kg feed	530.15±0.99[a]
	Garlen 2 gm./kg feed	520.25±1.74[a]
2nd week	Control	540.93±13.07[a]
	Garlen 1 gm./kg feed	459.18±4.19[b]
	Garlen 2 gm./kg feed	449.69±2.97[c]
4th week	Control	542.30±12.69[a]
	Garlen 1 gm./kg feed	425.48±8.63[b]
	Garlen 2 gm./kg feed	403.72±7.17[c]
6th week	Control	534.13±7.52[a]
	Garlen 1 gm./kg feed	420.00±0.95[b]
	Garlen 2 gm./kg feed	379.43±7.01[c]
8th week	Control	548.40±4.04[a]
	Garlen 1 gm./kg feed	387.60±2.51[b]
	Garlen 2 gm./kg feed	362.50±10.40[c]

*Number of fish = 3, for each week: Means within the same column of different letters are significantly different at (P<0.05).

6- **Effects of different treatments of Garlen® on Total bacterial count, Total Enterobacteriaceae count and Total Coliform count present in gut of *sea bream*:**

Data of Total bacterial, Total Enterobacteriaceae and Total Coliform counts were transformed to logarithmic transformation and analysis were take place on this logarithmic transformation. In zero day and 2nd week these bacterial counts showed no significant value. In 4th, 6th, and 8th weeks these bacterial counts decreased progressively in the groups treated with Garlen® immunostimulant (high dose then small dose) than the control group as shown in Table (6).

Table 5. Serum total protein, albumin, and globulin and albumin/globulin ratio among different groups in different weeks.

	Groups	Total protein(g/dl)	Albumin (g/dl)	Globulin (g/dl)	A/G ratio
		Mean ± Std. Error	Mean ± Std. Error	Mean ± Std. Error	Mean ± Std. Error
Zero day	Control	5.33±0.33[a]	2.67±0.33[a]	2.67±0.33[a]	1.06±0.24[a]
	Garlen 1 gm./kg feed	5.00±0.58[a]	2.67±0.33[a]	2.33±0.33[a]	1.14±0.15[a]
	Garlen 2 gm./kg feed	4.33±0.33[a]	2.67±0.67[a]	1.67±0.33[a]	2.00±1.00[a]
2nd week	Control	5.33±0.33[b]	2.67±0.67[a]	2.67±0.33[b]	1.06±0.39[a]
	Garlen 1 gm./kg feed	6.00±0.58[b]	2.67±0.67[a]	3.33±0.33[a]	0.80±0.18[a]
	Garlen 2 gm./kg feed	6.67±0.67[a]	2.33±0.33[a]	4.33±0.33[a]	0.53±0.04[a]
4th week	Control	4.67±0.33[b]	2.33±0.33[a]	2.33±0.33[b]	1.06±0.24[a]
	Garlen 1 gm./kg feed	7.10±0.15[a]	2.00±0.58[a]	5.10±0.44[a]	0.42±0.15[b]
	Garlen 2 gm./kg feed	7.57±0.03[a]	2.33±0.33[a]	5.23±0.32[a]	0.46±0.10[b]
6th week	Control	4.50±0.29[b]	2.33±0.33[a]	1.83±0.17[b]	1.28±0.15[a]
	Garlen 1 gm./kg feed	8.50±0.17[a]	1.57±0.18[a]	6.73±0.19[a]	0.23±0.04[b]
	Garlen 2 gm./kg feed	8.10±0.12[a]	1.37±0.12[a]	6.93± 0.32[a]	0.20±0.02[b]
8th week	Control	4.40±0.30[c]	3.00±0.10[a]	1.40±0.40[c]	2.84±1.24[a]
	Garlen 1 gm./kg feed	8.87±0.03[b]	1.30±0.06[b]	7.57±0.03[b]	0.17±0.01[b]
	Garlen 2 gm./kg feed	9.67±0.03[a]	1.27±0.03[c]	8.40±0.06[a]	0.15±0.00[b]

*Number of fish = 3, For each week: Means within the same column of different letters are significantly different at (P<0.05).

7- Effects of different treatments of Garlen® on (Antibody titer):

The antibody titration differed significantly among different treated groups at different weeks according to the effect of immunostimulant used. The higher antibody titers in general observed in the groups treated with Garlen® immunostimulant (high dose and small dose) than the control group as shown in Table (7).

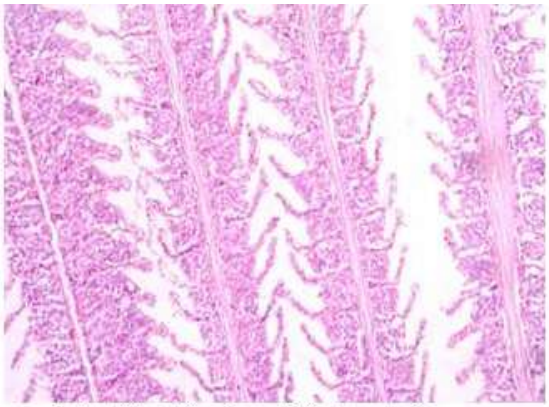
8- Effects of different treatments of Garlen® on mortality after challenge with *Vibrio alginolyticus* and relative level of protection:

The mortality level increased in the control untreated groups than the groups treated with Garlen® immunostimulant (both doses). Meanwhile, the relative level of protection showed higher level in Garlen® treated groups (60% in high dose and 50% in small dose) as indicated in Table (8)

Table 6. Logarithmic transformation of Total bacterial count, Total Enterobacteriaceae count and Total coliform count among different groups in different weeks.

	Groups	Total bacterial count	Total enterobacteriaceae count	Total coli form count
		Mean±Std. Error	Mean±Std. Error	Mean±Std. Error
Zero day	Control	3.48±0.00a	3.41±0.00a	3.11±0.00a
	Garlen 1 gm./kg feed	3.49±0.00a	3.26±0.00a	2.48±0.00a
	Garlen 2 gm./kg feed	3.46±0.00a	3.15±0.00a	2.70±0.00a
2 nd week	Control	3.51±0.00a	3.41±0.00a	3.11±0.00a
	Garlen 1 gm./kg feed	3.32±0.00a	3.26±0.00a	2.48±0.00a
	Garlen 2 gm./kg feed	3.28±0.00a	3.15±0.00a	2.70±0.00a
4th week	Control	3.50±0.02a	3.44±0.01ab	2.58±0.10a
	Garlen 1 gm./kg feed	3.26±0.01b	3.19±0.02c	2.42±0.06b
	Garlen 2 gm./kg feed	3.13±0.02c	3.08±0.02d	2.20±0.10c
6th week	Control	3.45±0.03a	3.41±0.03a	2.26±0.14b
	Garlen 1 gm./kg feed	3.18±0.02b	3.05±0.05b	2.59±0.06a
	Garlen 2 gm./kg feed	3.02±0.06c	2.90±0.09c	2.30±0.17b
8th week	Control	3.47±0.02a	3.44±0.01a	2.36±0.06b
	Garlen 1 gm./kg feed	2.99±0.11b	2.75±0.23b	2.52±0.04a
	Garlen 2 gm./kg feed	2.72±0.07c	2.59±0.06c	2.10±0.10c

*Number of fish = 3, For each week: Means within the same column of different letters are significantly different at ($P<0.05$).



Fg.1. Gills of Sea bream Fish in control group showed congestion in blood vessel of gill filaments hyperplasia of epithelial cells between secondary lamellae led to fusion.(H&E X 200)

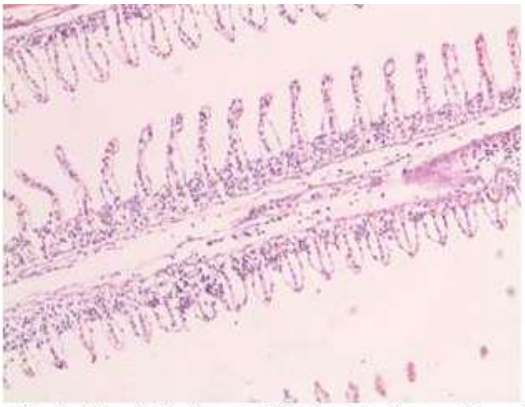


Fig. 2. Gills of Sea bream Fish in control group showed edema and lifting of the lamellar epithelium of secondary lamellae with numerous mononuclear leukocytic infiltration.(H&E X 200)

Table 7. Antibody titer (log_2) among different groups in different weeks.

	Groups	Antibody titer Mean ± Std. Error
first week	Control	2. 3333±.3333c
	Garlen 1 gm./kg feed	5.3333±.3333b
	Garlen 2 gm./kg feed	6.6667±.3333a
2 nd week	Control	2.6667±.3333c
	Garlen 1 gm./kg feed	5.6667±.3333b
	Garlen 2 gm./kg feed	7.3333±.3333a
3 rd week	Control	3.3333±.3333b
	Garlen 1 gm./kg feed	5.6667±.6667ab
	Garlen 2 gm./kg feed	5.3333±.3333a
4th week	Control	3.6667±.3333c
	Garlen 1 gm./kg feed	5.6667±.8819b
	Garlen 2 gm./kg feed	6.0000±.0000a

*Number of fish = 3, For each week: Means within the same column of different letters are significantly different at (P<0.05).

9-Histopathological examination:

Histopathological examination of different organs of fish in control group that challenged with pathogenic Vibrio alginolyticus only without Garlen® showed drastic changes ,where gills showed congestion in blood vessel of gill filaments, hyperplasia of epithelial cells between secondary lamellae led to fusion(Fig.1),edema and lifting of the lamellar epithelium of secondary lamellae with numerous mononuclear leukocytic infiltration(Fig.2).Gills of fishes fed on 0.1 ml/kg Garlen® showed congestion in blood vessel and moderate epithelium liftening of secondary lamellae.(Fig.3).While gills of fishes fed on 0.2 ml/kg Garlen® appeared normal with slight curved secondary lamellae(Fig.4). Liver of control group revealed congestion of central vein, hepatic sinusoids and vacuolar degeneration of hepatocytes, with infiltration by monocytes(Fig.5&6).Meanwhile liver of fishes fed on fed on 0.1 ml/kg Garlen® showed dilation , congestion of blood vessels and edema around hepatocytes, with proliferation of von kupffer's cells(Fig.7),but liver of fishes fed on fed on 0.2 ml/kg showed apparent normal hepatocytes.(Fig. 8).Spleen of control group revealed dilation of blood vessel, severe depletion of white bulb ,edema, and severe activation of melanomacrophage center.(Fig. 9& 10).While spleen of fishes fed on fed on 0.1 ml/kg Garlen®showed the same lesions with moderate severity(Fig. 11),but spleen of fishes fed on fed on 0.2 ml/kg Garlen® appeared with normal white and red bulb(Fig.12). Kidney of control group showed congestion of renal blood vessels, vacuolar degeneration or necrosis of renal tubules epithelium, and interrenal haemopoietic tissues with mononuclear cell infiltration, sometimes atrophied glomerular tuft and widening of bowman capsules space were observed(Fig.13&14).While kidney of fishes fed on fed on 0.1 ml/kg Garlen® appeared with congestion of renal blood vessels and moderate destruction of renal tubules(Fig.15),but kidney of fishes fed on fed on 0.2 ml/kg Garlen® showed congestion of glomerular tuft with slight destruction of renal tubules.(Fig.16).

Table 8. Mortality percent and Relative level of protection after challenge with pathogenic bacteria *(Vibrio alginolyticus*) among different treated groups.

Groups	N=70	Mortalities		Protected	
		No	%	No	%
Control (-ve)	10	10	100	0	0
Garlen 1gm./kg feed	10	5	50	5	50
Garlen 2gm./kg feed	10	4	40	6	60

Number of fish = 3, For each week: Means within the same column of different letters are significantly different at (P<0.05).

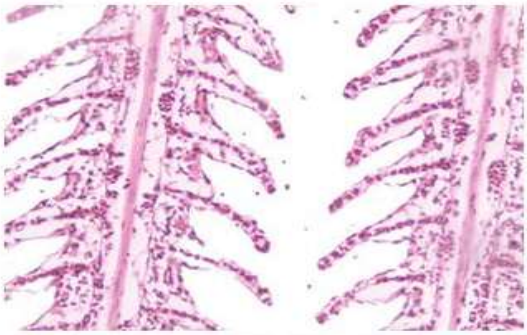


Fig. 3. Gills of Sea bream Fish fed on diet incorporated with *Garlen* 0.1 ml/ kg feed showed congestion in blood vessel and moderate epithelium liftening of secondary lamellae. (H&E X400).

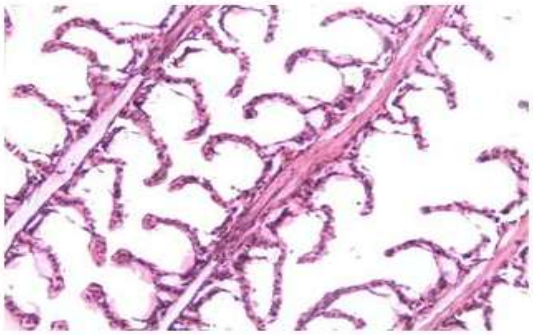


Fig. 4. Gills of Sea bream Fish fed on diet incorporated with *Garlen* 0.2 ml/ kg feed showed apparent normal gills with slight curved secondary lamellae.(H & E X 200)

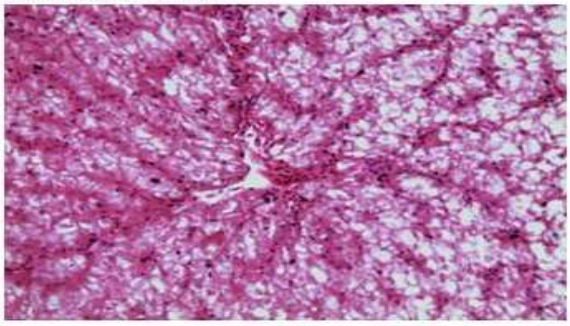


Fig. 5 Liver of Sea bream Fish in control group showed congestion of central vein and hepatic sinusoids with vacuolar degeneration of hepatocytes.(H &E X 200)

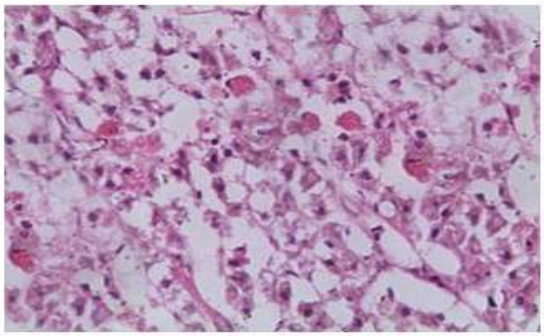


Fig. 6. Liver of Sea bream Fish in control group showed severe vacuolar degenerative change and infiltration by monocytes. (H & E X 400).

4. DISCUSSION

The involvement of allicin in stimulating immune parameters would certainly suggest that this compound may be involved with the beneficial effect of garlic. These immune parameters are the proliferation of differential leucocytes, mostly monocytes and thrombocytes. Thrombocytes, The main percent of neutrophils decreased significantly I n fish feed on diet containing 1,2gm /kg feed Garlin (table 1) also known as platelets, are considered to be innate inflammatory cells and as such are rapidly deployed to sites of injury or infections, where they potentially modulate inflammatory processes by interacting with other leucocytes implicated in immune responses, and secreting cytokines, chemokine's and other inflammatory mediators (Weyrich and Zimmerman, 2004). These results agreed with Fazlolahzadeh et al (2011) who revealed that there was a significant increase in leucocytic count and main percent of neutrophils decrease significantly in fish feed in diet containing 0.45 and 0.6 gm. /kg These results also agreed with those obtained by (Martins et al., 2002) who cited that addition of *Allium sativum* to fish diets increased the erythrocyte number, hemoglobin concentration, hematocrit value, leucocytes, and Thrombocytes. Mesalhy et al. (2008) these results also agreed with the results of histological examination of liver through the activation of kupffer's cells and melanomacrophage center. In addition to the previous, table (2) revealed that the groups received higher dose (2gm/kg feed Garlen®), showed increased significant value ($P<0.05$) in phagocytic assay than the groups received smaller dose (1gm/kg feed Garlen®), but also both doses showed increased significant value ($P<0.05$) in phagocytic activity and phagocytic index than the control group from the 2nd week till 8th week. Corzo-Martinez et al. (2007) reported that some of the protein-based sulphur compounds in garlic, such as S-allyl cysteine SAC, S-ethyl cysteine, N-acetyl cysteine, lectin and pectin, which are stable, odorless and bioavailable, may well be responsible for priming pharmacological and immunological effects.

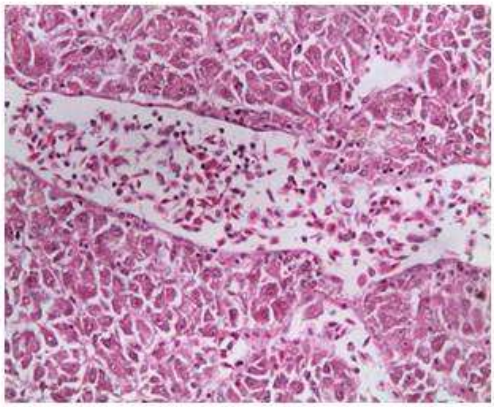


Fig.7. Liver of Sea bream Fish fed on diet incorporated with *Garlen* 0.1 ml/ kg showed dilation , congestion of blood vessels and edema around hepatocytes, with proliferation of von kupffer's cells.(H &E X 200)

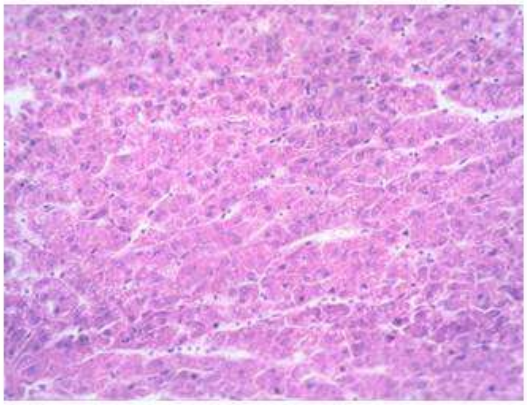


Fig.8. Liver of Sea bream Fish fed on diet incorporated with *Garlen* 0.2 ml/ kg showed apparent normal hepatocytes.(H &E X 200)

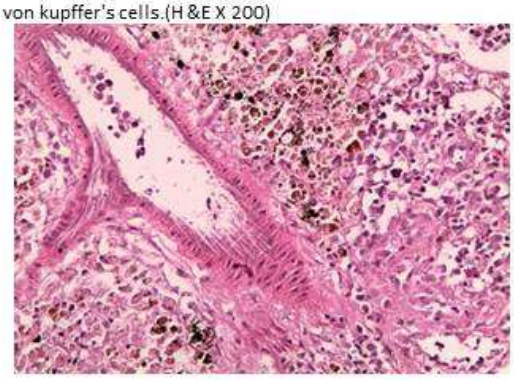


Fig. 9. Spleen of Sea bream Fish in control group showed dilation of blood vessel and hyperactivation of melanomacrophage center. (H&E X 200)

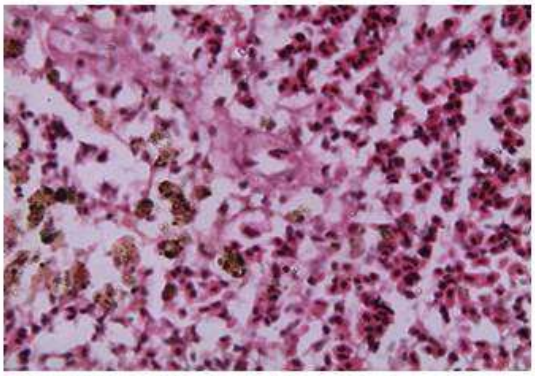


Fig. 10. Spleen of Sea bream Fish in control group showed severe depletion of white bulb and edema, with massive deposition of hemosiderin in the melano-macrophage centers (H&E X 400)

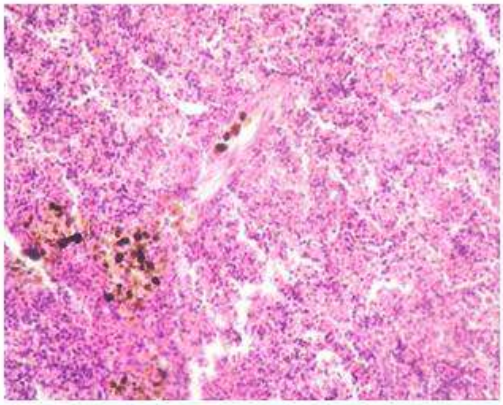


Fig. 11. Spleen of Sea bream Fish fed on diet incorporated with *Garlen* 0.1 ml/ kg showed moderate depletion of white bulb and activation of melanomacrophage center.(H&E X 200)

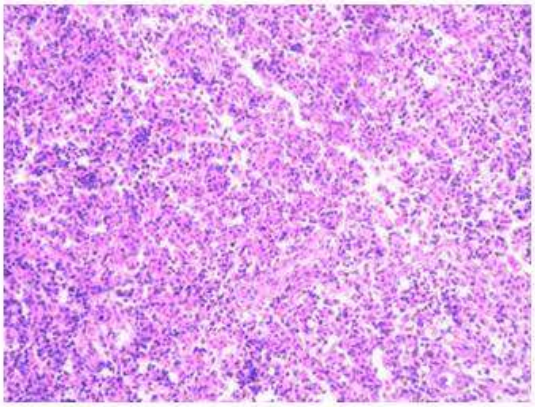


Fig.12. Spleen of Sea bream Fish fed on diet incorporated with *Garlen* 0.2 ml/ kg showed apparent normal white and red bulb.(H & E X 200)

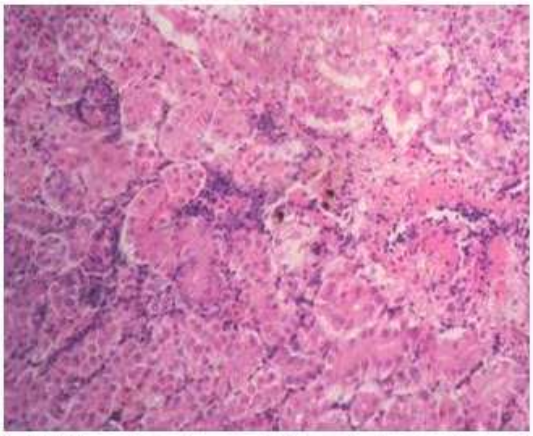


Fig.13. Kidney of Sea bream Fish in control group showed congestion of renal blood vessels ,necrosis of renal tubules epithelium, and interrenal haemopoietic tissues, with mononuclear cell infiltration (H &E X 200)

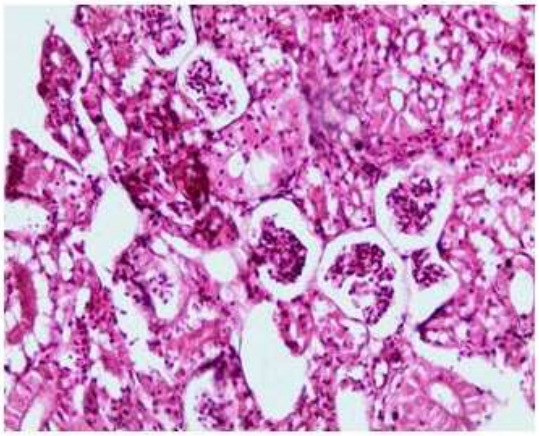


Fig. 14. Kidney of Sea bream Fish in control group showed vacuolar degeneration of renal tubules ,and atrophied glomerular tuft with widening of bowman capsules space (H &E X 200)

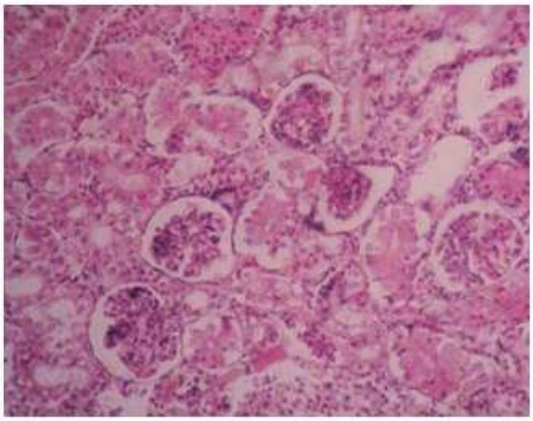


Fig. 15. Kidney of Sea bream Fish fed on diet incorporated with *Garlen* 0.1ml/ kg showed congestion of renal blood vessels and moderate destruction of renal tubules (H&E X 200)

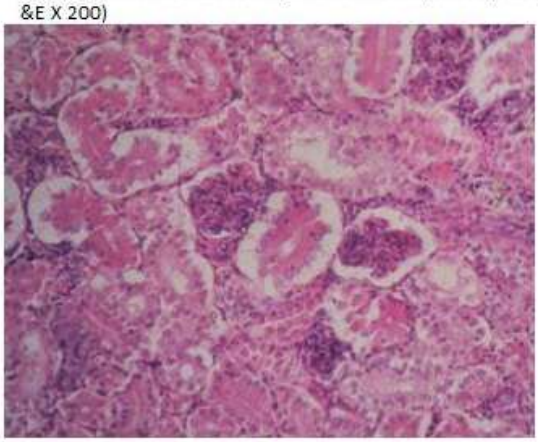


Fig.16. Kidney of Sea bream Fish fed on diet incorporated with *Garlen* 0.2 ml/ kg showed congestion of glomerular tuft with slight destruction of renal tubules . (H &E X200)

The involvement of allicin in stimulating immune parameters would certainly suggest that this compound may be involved with the beneficial effect of garlic. Nya and Austin (2009) revealed that dietary garlic enhances the non-specific immune mechanism of Rainbow trout by stimulating the proliferation of immune cells, and enhancing phagocytosis, oxidative burst. Lysozyme, one of the important bactericidal enzymes of innate immunity is an indispensable tool of fish to fight against infectious agents (Lindsay, 1986). As indicated in table (3) the groups treated with immune stimulant (Garlen®), revealed that the groups received higher dose 2gm/kg feed) showed increased significant value ($P<0.05$) in serum lysozome and bactericidal activity than the groups received smaller dose 1gml/kg feed), but also both doses showed increased significant value ($P<0.05$) in these parameters than other treated groups and the control group from the 2nd week till 8th week. Engstad et al. (1992) reported that immunostimulants can also increase serum lysozyme activity by increasing the number of phagocyte-secreting lysozyme, or by increasing the amount of lysozyme synthesized per cell. Lysozyme is found in a wide range of vertebrates including fish and is one of the defensive factors against invasion by microorganisms (Evelyn, 2002). Ndong and Fall (2006) reported that garlic at a concentration of 0.5 % over a 2-4 week period in juvenile hybrid tilapia improved lysozyme activity. Our results also agreed with the results obtained by Nya et al. (2010)

who found that a significant difference in serum lysozomal activity and serum bactericidal activity in the experimental groups compared with the controls. Allicin has been considered to be a transient compound, being rapidly decomposed or hydrolyzed into various sulphur-containing compounds. Furthermore, it has been argued that these breaks- down products of allicin exhibit the actual antibacterial and antifungal effects of garlic (Tansey and Appleton, 1975) and therefore increasing its bactericidal activity. The role of some immunostimulants in the anabolism after a prolonged period of administration was investigated. In the present study as indicated in tables (4) and (5), the groups treated with 0.2ml/kg feed (Garlen®) showed increased significant value ($P<0.05$) of serum total protein and globulin and decreased plasma cortisol level from the 2nd week to 8th week than the groups treated with small dose of Garlen® (0.1ml/kg feed) and also than other treated groups, serum albumin level and A/G ratio not revealed any significant value ($P>0.05$) in 2nd and 4th week. In 6th and 8th weeks serum albumin level and A/G ratio showed decreased significant value ($P>0.05$) in groups treated with Garlen® (both doses) than other treated groups and also than the control one which could be attributed to the immune-modulatory effect of garlic extract on the liver cells *which activate the anabolic capacity of the hepatocytes to produce blood proteins particularly globulin (Hussein 1996).*This high levels of cortisol reduce fish resistance against pathogenic organisms and the cause of this higher vulnerability could be the immunosuppressive effect of cortisol that seems to affect antibody production (Ellsaesser and Clem, 1986a, 1986b and Tripp et al., 1987) but this level of cortisol was significantly decreased ($P>0.05$) in groups treated with 2gm and 1gm/kg feed Garlen® (362.50±10.40f and 387.60±2.5e respectively)in 8th week Roshan ,et al (2010) found a significant decrease in Blood glucose ,Cholesterol , Triglycerides , and Cortisol these revealed that *allam sativum* has got significant anti stress activities at the same time increased leucocytic count, increasing the number of phagocytes or activating phagocytosis and serum globulin level in these treated groups. The results of total bacterial, total enterobacteriaceae and total Coliform counts showed that the groups treated with higher dose (2gm/kg feed) of (Garlen®), showed decreased significant value ($P>0.05$) in bacterial counts than the groups received smaller dose (1gml/kg feed), but also both doses showed decreased significant value ($P>0.05$) in bacterial counts than other treated groups and also than the control group in all weeks. Orzo-Martinez et al. (2007) reported that garlic can help in the control of pathogens, especially bacteria and fungi, and increase the welfare of fish. In addition, direct intragastric effects are feasible because Allium sativum antimicrobials are not affected by acid environments (Lawson, 1996); otherwise the gastric juice enhances the antimicrobial activity of Allium sativum constituents (Fortunator, 1995). The antibacterial action of garlic depends on allicin and is thought to be due to multiple inhibitory effects on various thiol-dependent enzymatic systems (Ankri and Mirelman, 1999).Garlic contains allicin, which promotes biogenic performance due to its positive effect on the intestinal flora, thereby improving digestion, availability of natural feed, supply of nutrients and utilization of energy which influences the growth of fish *(*Khalil et al., 2001). The fry showed reduced mortality, in the present study the relative level of protection of fish, challenged after 7 weeks of experiment was better than that of the control group. Garlic has been used for centuries in many societies against parasitic, fungal, bacterial and viral infections. The recent chemical characterization of their sulphur compounds has promoted claims that such compounds are the main active antimicrobial agents (Rose et al., 2005). Moreover, the high garlic dose (2gm/kg feed) afforded greater protection than the lower dose 1gm/kg feed (60% and 50% respectively). The survival rate and the relative level of protection were significantly greater in all garlic-supplemented groups when compared with the control group at the end of the experiment (8 weeks) as indicated in table (8). These

results also are in line with the observation of the results of antibody titer of these groups as indicated in table (7).Regarding the histopathological examination of different organs of sea bream challenged experimentally with *V.alginolyticus* at the end of 7th week(control group),gills showed congestion in, hyperplasia of epithelial cells between secondary lamellae led to fusion, edema and lifting of the lamellar epithelium of secondary lamellae with numerous mononuclear leukocytic infiltration, These gill alterations might harm the ionic changes in fish and constitute a portal of entry to other diseases (Moraes and Martins, 2004). Similar gill changes were observed by(Morrison et al., 2001 and Villamil et al., 2003). while liver of control group revealed congestion of central vein, hepatic sinusoids and vacuolar degeneration of hepatocytes, with infiltration by monocytes these alterations were incorporated with the decrease in the level of total protein and globulin in control group in 8th week as liver is an organ that may be used as an indicator of alterations in nutritional or physiological status as commented by (Segner and Juario 1986). However, general metabolism of fish is compromised in cases of infectious diseases. Similar results were recorded by (Korun and Timur 2009).Spleen revealed dilation of blood vessel, severe depletion of white bulb, edema, and hyperactivation of melanomacrophage center that may be due to rapid clearance and elimination of bacteria from the blood by macrophages which subsequently settle in the haemobiotic tissues which activate melanomacrophage centers. Later on, the macrophage that contain organisms may lyse and liberate bacterial toxins and result in excessive damage (Soliman, 1988 b).Our results agreed with (Rebort et al., 2012) .Moreover kidney showed congestion of renal blood vessels, vacuolar degeneration or necrosis of renal tubules epithelium, and inter renal haemopoietic tissues with mononuclear cell infiltration, sometimes atrophied glomerular tuft and widening of bowman capsules space .The kidney's alterations here observed were similar to the ones in turbot infected with *V. pelagius* (Villamil et al., 2003) and in turbot *Colistium nudipinnis* with septicemia caused by *V. splendidus* (Diggles et al., 2000)**.**Concerning the clinical picture of the vibriosis this may attributed to that V alginolyticus is capable of producing multiple virulent extracellular products (ECP) mainly protease, haemolysin and siderophore that might be responsible for the virulence of *V.alginolyticus* strains Gomez-Leon et al. 2005). Also, Balebona et al.,1998andJun et al., 2003attributed the pathological lesions produced by V alginolyticus in fish to the effect of ECPs especially their hydrolytic and hemolytic components were toxic and responsible for the invasive , proliferative processes of these bacteria and tissue damage. Our results also are in line with the observation of the results of histopathological examination of gill, liver ,spleen and kidney of *sea bream* challenged experimentally with *V.alginolyticus* and fed on diet incorporated with Garlen® which showed moderate to mild pathological lesions and enhancement of these lesions according to Garlen® doses(1and.2gm /kg)compared to the experimentally infected group in challenge test. Our results also agreed with that obtained by Meselhy et al. (2008) who reported that survival rates were significantly higher in all groups treated with garlic compared to the control and the relative level of protection against the challenge infection by *Aeromonas hydrophila* was higher in all treated groups than the control. Moreover our results agreed with Nya et al. (2010) who found that allicin has the ability for the control of *A. hydrophila* infection in Rainbow trout. (Tsao and Yin, 2001 and Bjarnsholt et al., 2005) reported that allicin has been reported to be inhibitory against bacterial pathogens of clinical significance including *Escherichia coli* and *Staphylococcus aureus.*

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