# Effect of Dietary Herbal Extract on *Tilapia Zillii* Challenged With *Photobacterium Damselae*

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**Abstract:**

The present study focused to evaluate Oregano on hematological parameters, plasma analysis and disease resistance of *Tilapia zillii*. In this experiment, fish were divided into 3 groups in triplicate manner. The first two groups fed onto basal diet, the third group fed onto basal diet supplemented with Dosto oregano oil 10% powder. Results revealed that oregano enhance the hematological parameters and plasma parameters than basal diet. In addition to immunocompetence assay against *Photobacterium damsale* subspecies *damsale*, the obtained results indicated that, oregano enhanced the immune responses and increased resistance to *Ph. damsale* infection in *T. zillii*.

**Introduction:**

Oregano, *Origanum* spp which, is a member of the Labiatae family of plants, indigenous to the Mediterranean region, one of natural products with potential to be used as growth promoters ***(Fukayama et al., 2005)***. There are many Oregano species as *O. vulgare* L. (oregano) and *O. majorana* L. (marjoram) ***(Baydar et al., 2004)***. *Origanum* spp have shown prominent results in inhibiting the growth of the bacteria ***(Souza et al. 2006 & Oliveira et al., 2009)***, fungi ***(Cleff et al., 2010)*** and the synthesis of the microbial metabolites ***(Burt et al., 2005).*** Also, they had digestive properties ***(Platel and Srinivasan, 2004)***. These properites are reported to their constituents as the main constituents are carvacrol and thymol ***(Burt et al., 2005*** and ***Zheng et al., 2009).*** Recently, it is used in fish ration for improvement the fish production, enhance immune system of fish against any microbial infection and as growth promoters in *Oreochromi niloticus* diet ***(Seden et al., 2009)*** who found improvement in growth and disease resistance to pathogens after challenge with *Aeromonas hydrophila*. Also, these properities concluded by ***Zheng et al. (2009)*** who used oregano in channel catfish and ***Abdel-Latif and Khalil (2013)*** who used commercial product of oregano essential oils on cultured Nile tilapia (Ropadiar). *Photobacterium damselae* subsp. *damselae* is a marine bacterium, its taxonomic status within the family

Vibrionaceae ***(Shieh et al., 2003)*** has changed repeatedly .After its original description as *Vibrio damselae*, It was subsequently transferred to the genus *Photobacterium* on the basis of phenotypic data ***(Smith et al., 1991)***, and further support was obtained from the phylogenetic analysis carried by ***Ruimy et al. (1994)***. It is considered a primary pathogen of several species of wild fish as well as of fish species of economic importance in aquaculture .Cultivated species reported to be affected by this pathogen include *S. aurata* ***(Vera et al., 1991)***, *D. labrax*, yellowtail, *P. auriga*, white seabream, ***(Labella et al., 2010),*** *Mugil cephalus*, *M. capito* and Nile tilapia ***(Reyad and Salah, 2008)***. The recent first reports on isolation of this pathogen from diseased marine fish of new cultured species, suggest that *Photobacterium damselae* subsp. *damselae* can be considered as an emerging pathogen in marine aquaculture ***(Labella et al., 2011)***. It may also be pathogenic to mammals, including humans by infecting wounds and possibly leading to fatalities ***(Aigbivbalu and Maraqa, 2009)***. The aim of this study is, trial for using the oregano as supplementation in diet of *Tilapia zillii* challenged by *Photobacterium damselae* subsp. *damselae*.

**Materials and Methods**

**Fish :**

A total number of 90 apparently healthy *Tilapia zillii* with an average body weight of 13 ± 1.5 g were obtained from Fish Research Center at Suez Canal University.

**Diet:** Fish were fed commercial ration (purina aquamax pond fish) which guaranteed analysis for percentages of Crude protein 32%, Crude fat 3%, Crude fiber 5%, Ash 11% ,Calcium (2-3)% Phosphorus 1.10% and Sodium 0.6 % provided daily at 3% of body weight divided into two amounts at all the period of experiment according to ***Liu et al. (2011)***.

**Drug :**

Dosto mineral powder with 10% oregano oil (250 g per one Ton) to the basal diet of experimental fish during the period of experiment. It composed of 10 g (60% carvacol +1% thymol), 44g 1,2 propandiol as carrier, 30 g glycerine-polyethylenglycol-Ricinoleate as emulsifier and purified water up to 100 g.

**Experimental design and diets**

A total of 90 fish of *Tilapia zillii* of an average body weight 13 ± 1.5 g were equally distributed into three groups . Each group consisted of 30 fish each subdivided into 3 subgroups (10 fish/sub group) in glass aquaria 100 L with water daily changed and aerated through aerator at 12 hr light /12 hr dark period at water temperature fluctuated from 25 ± 2ºC. Fish were acclimated for 2 weeks in aquaria. The first and second groups served as a control group, fed into basal diet of 4000 kcal/kg digestible energy and 32% protein twice daily at 3% feeding

rate. The third groups fed twice daily at 3% feeding rate for 2 weeks into the same basal diet with addition of Dosto powder extract with concentration of 0.25 g/ Kg of diet by addition of vegetable oil in the pellet for coating and mixing the ration well manually.

**Immunocompetance test (disease resistance)**:

After the end of acclimation period onto the basal diet and supplemented diet , a challenge test was performed on each group with well identified *Photobacterium damsale* subspecies *damsale* kindly supplied from Fish Diseases & Management Dept., Fac. Of Vet. Medicine, Suez Canal University. Bacteria were cultured on BHIA 3% NaCl for 18 hr then the pure cultures were harvested and suspended in 0.9 % sterile physiological saline solution. The concentration of bacteria was adjusted to McFarland turbidity standard corresponding to $5.2 \times 10^5$ CFU /ml according to **Khouadja *et al.* (2014)** by the optical density of suspension. The first group was injected intraperitoneally with 0.1 ml of a bacterial suspension .The second group injected with 0.9% sterile physiological saline solution. The third group fed into supplemented diet, injected also with 0.1 ml of a bacterial suspension intraperitoneally. Mortality and morbidity rates were monitored daily for one week after injection .Bacterial re-isolation from experimentally infected dead fish was tried.

**Determination of haematological paramaters and plasma analysis:**

After acclimation period onto the diet in all groups, six fish of all groups were sampled for blood indices for differentiation between basal diet and supplemented diet on the fish blood. After injection, blood collected from fish from each injected group in third day after injection for evaluation the effect of Oregano onto the non-specific innate immunity of fish .Day 3 was chosen as sample time as the previous studies have shown that the 3rd day showed peak levels of bacterial growth according to ***Raida and Buchmann (2009)***. All blood was collected by heart puncture of the fish using heparinized syringe into small sterilized vials containing anticoagulant (Cal heparin). It was used for examination of Erythrocytic count (RBCs) according to ***Shah and Altindag (2004),*** total leukocyte count (TLC) according to ***Schaperclaus (1992)*** using Giemsa stain, differential leukocyte count (DLC) according to ***Stoskopf (1993)***, Haemoglobin content (HB) and Haematocrite value (Ht%) according to ***Van Kampen and Zijlstra (1961)***. Plasma was collected after centrifugation (3600 g for 5 min) and stored in the deep freezer and used for examination of plasma total proteins, albumin, globulins

and A/G ratio according to ***Ericsson and Nister (2011)***.

**Statistical analysis**:

All results were expressed as means ± standard deviation (SD). Data from comparsion between basal diet and supplemented one were presented as fold change levels (means ± SD), calculated by dividing each parameter value from fish i.p. injected with *Photobacterium damselae* sub species *damselae* by the mean value from control fish, i.p. injected with saline, minus one. Fold values higher than 0 express an increase and lower than 0 a decrease in the parameters assessed relative to fish i.p. injected with saline. Data as well as fold change were analyzed by one-way ANOVA. Data were treated statistically according to **Zar (1999)**. The level of significance used was $P \leq 0.05$ for all statistical tests.

**Results:**

**Hematological parameters and plasma analysis of experimental fish:-**

As shown in Table (1) before infection, hematological assays data as a response to *Tilapia zillii* fed onto basal diet supplemented with oregano oil 10% 0.25g/ Kg .The concentration of HB and the number of RBCs were tended to be significantly increased in the experimental fish fed supplemented diet. As well as, this increase was accompanied by a significant increase in HCt. In addition, the total number of WBCs tended to be non-significantly increased than basal groups. Also, lymphocytes percentages showed no change in both groups. Besides, there were no significance increased in percentage of neutrophils ,monocytes and eosinophils. While the significance increased in percentage of basophils in basal diet than in supplemented diet. After infection, the concentration of hemoglobin (HB) was tended to be decreased in the experimental infected fish than the control groups. While, the number of RBCs were significantly decreased than control groups. This decrease was accompanied by a significant decrease in Hct than the control groups. In addition, the total number of WBCs tended to be significantly increased than control groups. The lymphocytes percentages showed significant lymphocytopenia than the control groups. On the other hand, there was significance increase in percentage of neutrophils and monocytes. While slightly decrease in eosinophils and basophils. Fold change data of the hematological parameters of infected fish fed onto supplemented diet with oregano in compared with infected fishes fed onto basal diet, The concentration of HB and the number of RBCs were significantly increased than basal diet group. This increase was accompanied by a significant increase in Hct. In addition, the total number of WBCs, neutrophils,

monocytes, basophils were tended to be significantly increased than the basal diet groups.

**Plasma analysis:** As shown in **(Table 2),** before infection, the total proteins of fish fed onto oregano diet, plasma albumin and globulins were significant increased than the control basal groups. After infection, infected fish fed onto basal diet total proteins which represented in plasma as albumin and globulin were significantly decreased than control groups. Fold change data of the total proteins, plasma albumin and globulins were significantly increased than the control basal groups.

**Immunocompetance test (disease resistance)**:

After 2 weeks of feeding, fish were challenged with *Photobacterium damsale* subspecies *damsale* and cumulative mortality % was recorded for one week. Fish mortality began from the first day post injection and continued until the 7rd day with mortality rate 63.33% in the infected group fed onto basal diet without supplementation while others two groups (control group and third group infected and fed onto supplemented diet) were 3.33%. As shown in **Table (3)**.

The clinical picture of the experimentally infected fish were nearly similar in all treatments including control but varied in the severity of the developed lesions. They showed signs of reduced appetite, lethargy, dark pigmentation and abnormal swimming behavior before death, hemorrhage on the external body surface, and bitten fins**.** Internally, the postmortem changes were distended intestine, abdominal fluid in peritoneal cavity, congested liver and congested gills. Also, showing congested kidneys and petechial hemorrhage on liver .Others showing pale liver with hemorrhage in abdominal cavity. As shown in **Plate (1)**.

**Table 1:** Showing Hematological parameters of experimental fish.

| Blood parameter | Before infection | | After infection | | | |
|---|---|---|---|---|---|---|
| | Mean ± Standard Deviation | | Mean ± Standard Deviation | | Mean ± Standard Deviation | |
| | Supplemented diet (Or) | Basal diet (Ba) | Control Groups (Ba) | Infected Groups (Ba) | Fold change Supplemented diet (Or) | Fold change basal diet (Ba) |
| HB (g/dl) | 12.32 ± 1.10* | 6.92 ± 0.97 | 6.614 ± 0.95 | 6.32 ± 1.4 | 0.19 ± 0.07* | -0.044 ± 0.03 |
| HCT (%) | 41.45 ± 2.34* | 20.7± 2.43 | 19.085 ± 3.33* | ± 0.8216.4 | 0.85 ± 0.14* | -0.11 ± 083 |
| RBCS ($10^6$/µm$^3$) | 4.75 ± 0.43* | 2.87 ± 0.22 | 2.528 ± 0.43* | 1.98 ± 0.23 | 0.43 ± 0.17* | -0.214 ± 0.08 |
| MCV (FL ) | 28.5 ± 1.04 | 78.86 ± 8.86 | 79.028 ± 13.87 | 76.428 ±12.52 | -0.03 ± 0.028 | -0.22± 0.05 * |
| MCH (pg) | 88.52 ± 1.25* | 28.27± 0.898 | 25.871 ± 2.923 | 27.094 ± 1.707 | 0.07 ± 0.09 | 0.047 ± 0.07 |
| MCHC (g/dl) | 35.25 ± 0.25* | 34.3 ± 1.75 | 33.875 ± 2.542 | 32.94 ± 2.139 | 0.01 ± 0.02 | -0.08 ± 0.004 |
| WBCs ($10^3$/µm$^3$) | 7.4 ± 0.393 | 6.58 ± 0.76 | 6.1 ± 1.325 | 6.938 ± 0.877* | 2.37 ± 0.03* | 0.137 ± 0.02 |
| Neutrophils (%) | 8 ± 0.707 | 7.66 ± 0.942 | 7.428 ± 2.050 | 15.4 ± 9.537* | 2.01± 0.58 * | 1.075± 0.19 |
| Lymphocytes(%) | 87.75 ± 1.29 | 88.34 ± 0.829 | 88.88 ± 3.585* | 80.4 ± 2.244 | -0.034 ± 0.02 | -0.095 ± 0.01 |
| Monocytes (%) | 2.75 ± 0.433 | 2.5 ± 0.5 | 1.57 ± 1.049 | 2.56 ± 1.673* | 1± 0.35* | 0.630 ± 0.29 |
| Eosinophils (%) | 1.25 ± 0.433 | 1.5 ± 0.5 | 1.42 ± 0.5 | 0.64 ± 0.8 | -0.16 ± 0.28 | -0.54 ± 0.28 |
| Basophils (%) | 0.25 ± 0.433 | 0.57 ± 0.728* | 0.7 ± 0.9 | 0.4 ± 0.49 | -1± 0 | -0.42± 0.73 |

(*)Asterisk for significant differences between the same rows of two groups.

**Table 2:** Showing plasma analysis of experimental fish**.**

| Plasma parameters | Before infection | | After infection | | | |
|---|---|---|---|---|---|---|
| | Mean ± Standard Deviation | | Mean ± Standard Deviation | | Mean ± Standard Deviation | |
| | Supplemented diet (Or) | Basal diet (Ba) | Control groups | Infected Groups | Fold change Supplemented diet (Or) | Fold change basal diet (Ba) |
| Total protein (g/dl) | 3.48 ± 0.375* | 2.925 ± 0.1 | 3.31 ± 0.832* | 1.748 ± 0.043 | 1.09 ± 0.16* | 0.47 ± 0.09- |
| Albumin (g/dl) | 1.575 ± 0.08* | 1.15 ± 0.11 | 1.371 ± 0.522* | 0.92 ± 0.116 | 0.16± 0.06* | -0.32 ± 0.11 |
| globulin (g/dl) | 1.88 ± 0.14* | 1.23 ± 0.20 | 1.94 ± 0.843* | 0.828 ± 0.712 | 1.75 ± 0.26* | -0.57 ± 0.74 |
| A/G ratio | 0.83 ± 0.128 | 0.93 ± 0.213 | 0.71 ± 1.925 | 1.11 ± 0.191 | 0.088 ± 0.011 | 0.573 ± 0.06 |

* Asterisk for significant differences ($P \leq 0.05$) between the same rows between two groups.

**Table 3:** Showing the mortality rate of injected *Tilapia zillii* with *Photobacterium damsale* subspecies *damsale* during the experimental infection period.

| Groups | NO. | Mortality /day | | | | | | | Mortality % |
|---|---|---|---|---|---|---|---|---|---|
| | | 1st | 2nd | 3th | 4th | 5th | 6th | 7th | |
| **First group** | 30 | 8 | 5 | 3 | 0 | 2 | 0 | 1 | 63.33 |
| **Second group** | 30 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 3.33 |
| **Third group** | 30 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 3.33 |

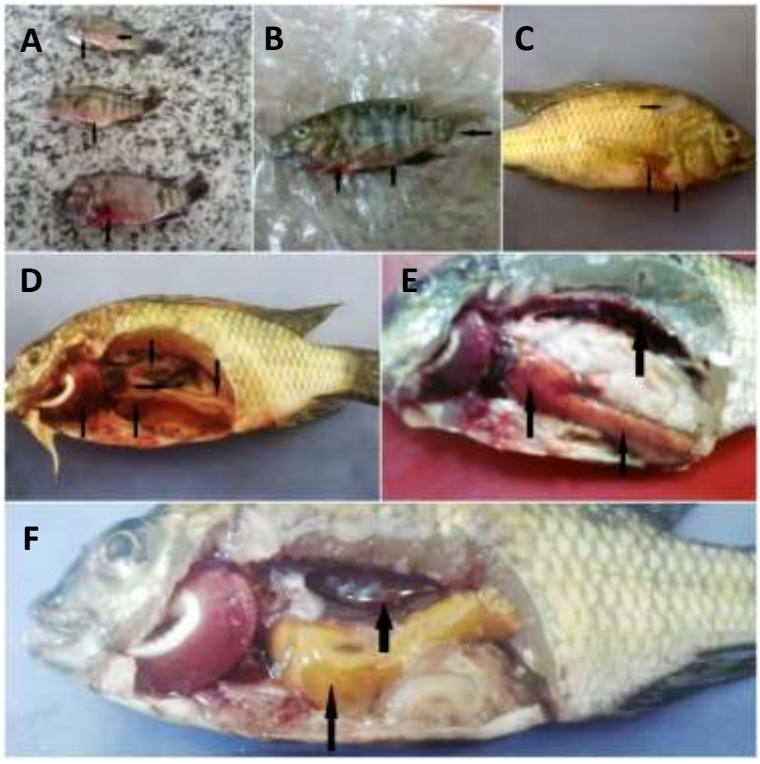


**Plate 1:** Experimentally infected *T. zillii* with *Photobacterium damsale* subspecies *damsale* showing **(A)** haemorrahage on the external body surface with slight ascitis in second fish.**(B)** showing haemorrhage at site of inoculation and anal fin with bitten caudal fin. **(C)** showing haemorrhage around detached scales area and at the base of pectoral fin. **(D)** showing distended intestine , abdominal fluid in peritoneal cavity ,splenomegaly, congested liver and congested gills.**(E)** showing congested kidneys and petechial hemorrhage on liver.**(F)** showing pale liver and haemorrhage in the abdominal cavity

**Discussion:**

During the last decade there was an increasing interest in the modulation of the nonspecific immune response of fish to elevate the general defense barriers and hence increase resistance against diseases through use of immunostimulants ***(Raa, 2000 and Sahoo and Mukherjee, 2002)***. In this study, the effect of addition of oregano in fish diet revealed that hemoglobin HB, RBCs and HCt were tended to be a significant increase in the experimental fish fed supplemented diet. These results agreed with ***Kumar et al. (2014)*** who found increasing in the hematological parameters (HB and RBCs) of experimentally infected catfish fingerlings fed onto herbal medicine as (*Ocimum tenuiflorum*, *Zingiber officinale* and *Allium cep*), so these results may attributed to age of the fish (early fingerling), higher activity and biological demand needed extra dissolved oxygen as mentioned by ***Wilhelm et al. (1992)*** *and* ***Walsh & Lue (2004).*** In addition, the total number of white blood cells (WBCs) tended to be non-significance increase with no change of the lymphocytes percentages. On the other hand, there were no significance increase in percentage of neutrophils and monocytes and eosinophils. These results agreed with ***Pourmoghim et al. (2015)*** who found non significance difference in the hematological parameters of rainbow trout fed onto supplemented diet with *Origanum vulgare* extract. Also, the total proteins which represented in plasma albumin and globulins were significantly increased. These results agreed with ***Ahmad et al. (2009)*** who found that total protein, albumin, and globulins levels increased significantly ($P \leq 0.5$) with *Origanum vulgare* extract, reaching the highest value at 0.5 % of the diet, as compared to the control. Moreover, the measurement of total proteins, albumin and globulins in serum or plasma are of considerable diagnostic value in fish, as it affects the general nutritional status, as well as the integrity of the vascular system and liver function as said by ***Schäperclaus et al. (1992).*** Serum or plasma proteins are various humoral elements of the non-specific immune system, measurable total protein, albumin and globulin levels suggest that high concentrations are likely to be a result of the enhancement of the non-specific immune response of fish. Concerning the experimentally infected *Tilapia zillii* with intraperitoneal injection with 0.1 ml of a bacterial suspension of *Photobacterium damsale* subspecies *damsale* at approximately 5.2 $X10^5$ CFU/ ml showed abnormal clinical signs as reduced appetite, lethargy, dark pigmentation and abnormal swimming behavior before death and hemorrhage in the first day post injection. Internally, there were congested liver, gills and kidneys. Thus, it was attributed to the lethal

and toxic effect of the bacteria as it is highly pathogenic, with 50% lethal doses (LD50) ranging from $10^3$ to $10^6$ colony forming units (CFU) per fish ***(Magariños et al. 1992).*** Fish mortality began the first day post injection and continued until the 7rd day with mortality rate 63.33%, this result agreed with ***Essam et al. (2016)*** who re-isolated *Ph. damsel*ae from seabass fingerlings infected with *Ph. damselae* and mortality rate reached to 60%, that indicated highly virulent of these species as mentioned by ***Pedersen et al. (2009)*** who mentioned strains that had haemolytic activity and those with ability to adhere to mucus seemed to be the most pathogenic. Concerning the hematological parameters of experimentally infected *Tilapia zillii* fingerlings revealed that, the concentration of hemoglobin, RBCs and HCT which tended to be decreased in the experimental fish than the control group. This result agreed with ***Chen et al. (2004)*** who found decreased in HCT of Nile tilapia infected with *V.vulnificus*. Also, agreed with ***Harbell et al. (1979); Barham et al. (1980) and Pathiratne & Rajapakshe (1998)*** who found decreasing RBCs, hemoglobin and hematocrit in chum salmon infected with *V. anguillarum*, in rainbow trout infected with *Aeromonas/Streptococcus* and in cichlid fish with epizootic ulcerative syndrome. They attributed their results to, RBCs contains hemoglobin, an iron containing protein, which facilitates transportation of oxygen by reversibly bonding with this respiratory gas thereby increasing its solubility in blood. The PCV percentage, hemoglobin and RBCs are good indicators for oxygen transportation capacity of fish, thus making it possible to establish relationship with the oxygen concentration available in the environment and the health of the fish. In the present study, the reductions in the total red blood cell count, PCV percent and hemoglobin rate indicates that the oxygen carrying capacity of the fish was impaired by the bacteria. In addition, the total number of WBCs tended to be increased than control groups, but the lymphocytes percentages showed lymphocytopenia than the control groups. On the other hand, there were increase in percentage of neutrophils and monocytes than the control groups, these results agreed with ***Essam et al. (2016)*** which indicated that, the lymphocytes have failed to interact with bacteria then the immune system start to increase the phagocytic cells to destroy and engulf the bacteria. ***Maule and Schreck (1990)*** found that, the lymphopenia which was commonly observed in many cases of acute stress in fish, was probably the result of a redistribution of these cells, which tended to be accumulated in certain organs, such as the thymus and the kidney. While

eosinophils and basophils were slightly decrease in experimented fish than the control groups as mentioned by ***Tavares-Dias and Moraes, (2003)*** that these cells were the least frequent type of leukocyte in the circulation of 11 teleosts. Regarding to total protein which represented in plasma albumin and globulins were decreased. This result agreed with ***Essam et al. (2016)*** who found slight decrease in total protein, globulin and albumine content of seabass fingerlinges experimentally infected with *P.damselae* sub. *piscida* and attributed this to failure in liver functions which indicated the degenerative changes that happened in the internal organs. Also similar results were reported by ***Maqsood et al. (2009)*** after I/P injection of *Cyprinus carpio*

Regarding Fold change data of the hematological parameters as a response to *Tilapia zillii* with intraperitoneal injection by identified *Photobacterium damselae* revealed that concentration of HB**,** RBCs and Hct tended to be significantly increased in the experimental infected fish fed supplemented diet than the fish fed with basal diet , these results agreed with ***Kakoolaki et al. (2016)*** who found increasing of HB, RBCs and HCt values in *M. cephalu*s challenged with *P. damselae* feed on supplemented diet with *C. sinensis* extract. In addition, the total number of WBCs**,** neutrophils and monocytes tended to be significanctly increased, these results agreed ***Kakoolaki et al. (2016)*** who concluded that these increase in numbers due to these types of cells are the major phagocytic cells responsible for phagocytosis, which is an indicator of immune response as neutrophils are components of the immune system that form the first line of defense against invasive agents and function as phagocytosis. Under acute stress an increase in innate immune response has been described by ***Vazzana et al. (2002),*** monocytes are cells with phagocytic function and elimination of bacteria. They are precursors of macrophages and are considered essential cells for life ***(Feldman et al., 2000)***. But the lymphocytes percentages showed non-significant decreased (lymphocytopenia), these results may attributed to that the decrease in lymphocytes is due to redistribution of circulating lymphocytes, with sequestration in lymphoid tissues ***(Mazeaud et al., 1977 and Rijnberk & Mol ,1997)***. Also, the total protein which represented in plasma albumin and globulins were significant increased. These results were indications of immune system enhancement, as mentioned by ***Pourmoghim et al. (2015)*** who suggested that measurable total proteins, albumin and globulins levels suggest that high concentrations are likely to be a result of the enhancement of the

non-specific immune response of fish.

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**الملخص العربى**

ركزت هذه الدراسة على تقييم الزعتر على قياسات الدم وتحليل البلازما وعلى مقاومة المرض فى الشبار الاخضر . فى خلال هذ التجربة قسمت الاسماك الى ثلاثة مجموعات .ولقد تغذت اول مجموعتين على غذاء رئيسى اما المجموعة الثالثة قد تغذت على مكمل غذائى 10% زعتر الدستو البودرة. ولقد أوضحت النتائج ان الزعتر يحفز قياسات الدم والبلازما عن الغذاء الرئيسى بالاضافة الى ان نتائج الاصابة بميكروب الفوتوبكتيريا دامسيلا أوضحت ان الزعتر يحفز الرد المناعى الاولى ويزيد مقاومة الاسماك ضد هذا الميكروب.