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Spirulina platensis and *Nigella sativa* as biocontrol of some bacterial diseases in *Oreochromis niloticus*

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Abstract

This study was conducted to evaluate the effect of *Spirulina platensis*, *Nigella sativa* and both of them at rate (7.5g/kg ration, 0.5g/100g ration and mix of them) respectively as feed additive for 8 weeks on growth performance, blood picture and immune system in *Oreochromis niloticus* (*O. niloticus*) before and after challenge with *A.hydrophila* .and resistance against *Aeromonas hydrophila* and *Streptococcus dysgalactiae* The results indicated that the addition of *Spirulina* or *Nigella* to diet improve growth performance, health condition, enhancement cellular and humoral immune responses, specifically (phagocytic activity, lysozyme, nitric oxide, total protein and its fraction especially γ globulin) either before and after challenged tests and increase resistance against bacterial infection. The combination of *Spirulina platensis* and *Nigella sativa* showed the highest values of growth performance, immunological parameters and give the lowest mortality percent against bacterial infection.

Key words: *Spirulina Platensis, Nigella Sativa, Oreochromis niloticus*, growth performance, fish immunity
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Introduction:

Fish farming industry depends on balance between health and growth condition of fish. The use of chemotherapy and antibiotics to combat fish diseases induce risk of generating resistant pathogen as well as bioaccumulation and environmental pollution, which are the major problems causing heavy losses especially bacterial diseases, also commercial vaccines are expensive for fish farming and are specific against particular pathogens (**Kumar *et al.*, 2005**). Immunostimulants are valuable for the prevention and control of fish diseases in aquaculture (**Mukesh *et al* .,2012**). There has been heightened research in developing new dietary supplementation strategies in which various health, immunostimulants and growth promoting compounds as probiotic, prebiotic, synbiotic, phytobiotic and other functional dietary supplements (**Denev, 2008**). *Spirulina platensis (SP)* is a photosynthetic, filamentous, blue-green microalgae and is generally regarded as a rich source of vitamins, essential amino acids, minerals, essential fatty acids (ϒ-linolenic acid) and antioxidant pigments such as carotenoids and phycocyanin. *Spirulina* is a rich source of protein (60–70%), so algae gained attention as a possible alternative protein source for cultured fish, it has anti-oxidant effects , antimicrobial effect , improved the growth performance, improved immunity and disease resistance in *Oreochromis niloticus*, (**Beresto, 2001; Bermejo *et al.*, 2008; Sherif *et al.*, 2012**; **Mai *et al.*, 2013** and **El-Sheekh *et al.*, 2014**). The other immunostimulants in the scope of attention is *Nigella sativa.* It is herbaceous plant belonging to family *Ranunculaceae.* This herb is widely spread in the Mediterranean counteries and in

Asia. India, Pakistan, East Africa, and middle Europe. It is known as black cumin or black seed and it cultivated for its seeds, which are used for different medicinal purposes as antimicrobial effect (**Hanafy and Hatem, 1991**). Some researchers have used black cumin seeds as enhancer for performance, growth and immune system of some fish species (**Abd Elmonem *et al.*, 2002; John *et al.*, 2007; Diab *et al.*, 2008 and Dorucu *et al.*, 2009**).

The current study was planned to evaluate the effect of *Spirulina platensis* or *Nigella sativa* and combination between them on the growth performance and immunological response before and after bacterial challenge in *Oreochromis niloticus*

Materials and Methods:

2.1. *Immunostimulants:*

Spirulina platensis (SP) algae: commercial Spirulina algae powder was obtained from international center for vital energy.*Nigella sativa* (*NS)*seeds:_ Commercial Nigella sativa seed was obtained from the market in pure form free from debris and other plant seeds.

2.2. *Bacterial strains*:

The microorganisms used in challenge test were kindly obtained from Microbiological Unit, Dept. of Fish Dis., Animal Health Res.rch Institute, Dokki. Well identified ***Aeromonas hydrophila*** and ***Streptococcus dysgalactiae***).

2.3. *Fish sampling:*

One hundred and sixty apparently healthy *O. niloticus* weighing 50±10 g collected from a private farm at Eltal Elkbir-Sharkia governorate. Were stocked in 8 glass aquaria (20 fish/aquarium). The fish were acclimated for 2 weeks and fed on commercial pelletized food 25% protein twice daily at 5% of their body weight. They were maintained in aerated, de-chlorinated tap water. The water temperature was adjusted at 25°C during the experiment. Fecal matters were siphoned out once daily and water was changed every 3 days to maintain a good water quality.

2.4. *Diet preparation:*

standard commercial fish diet pelletized was mixed with feed additive by using molas as binding material; fish were fed for 2 months. The experimental design is shown in table (1).

Table (1): Experimental outline of *O.nilticus* groups.

Fish group	Feed additive for 8 weeks			Challenge test 7days	
	Numbers of fish	Treatments	dose	No. of fish	bacteria
A	20	*Spirulina platensis*	7.5g/kg ration	20	*A.hydrophila*
	20			10	*S. dysgalactiae*
B	20	*Nigella sativa*	0.5g/100g ration	20	*A.hydrophila*
	20			10	*S. dysgalactiae*
C	20	*Spirulina platensis+ Nigella sativa*	7.5g/kg+0.5g/100g	20	*A. hydrophila*
	20			10	*S. dysgalactiae*
Control	20	Basal diet		20	*A.hydrophila*
	20			10	*S. dysgalactiae*
				10	Control negative

2.5. *Growth performance*:

It was calculated as following:

- **Body weight gain:** Final fish weight (g) – Initial fish weight (g) according to **Annet, (1985).**

Specific Growth Rate %: It was calculated as the percentage increase in weight per fish per day as suggested by **Pouomonge and Mbongtang (1993)**, using the following equation: **SGR% = (Ln WT – Ln Wt) / (T-t) x 100.**

2.6. Hematogram , serum biochemistry and immunological measurements :

Blood samples were collected at the end of experiment and after 7 days post challenge test, blood samples were collected on anticoagulant either 100 IU/ml sodium heparin for phagocytic activity or EDTA for estimation of haemogram, Another blood samples were collected without anticoagulant for serum separation to be used in biochemistry and immunological measurements.

2.6.1. Hematogram:

Erythrocytes and leukocytes were counted according to **Kanacu (1985)** using a haemocytometer , hemoglobin concentration was determined by acid hematin method according to **Coles(1986),** Packed cell volume PCV was determined by microhaematocrit centrifuge according to **Decie and Lewis (1991).**

2.6.2. Biochemical analysis:

Total protein was carried according to biuret methods as described by **Gornall *et al.*, (1949),** serum albumin was estimated by colometric methods according to **Doumas *et al.*, (1971)** using commercial kit, globulin was estimated by this equation (total protein- serum albumin) according to **Coles (1986),** A/G ratio was calculated from albumin present in serum in relation to the amount of globulin. Serum protein electrophoretic pattern was carried according to **Davis (1964)** and electropherogram was traced densitometrically by using **synGene- Gene Tools serial No. 17292*14518*sme,** Serum ALT and AST activities were estimated calorimetrically using Vitro Scient kits as described by **Reitman and Frankel (1957).**

2.6.3. Immunological measurement:

2.6.3.1. Nitric oxide assay (reactive nitrogen species): Fifty microliter of serum was added on an equal volume of Griess reagent in flat-bottomed 96-well plate, followed with gentle shaking. The plate was kept in dark room for 15 min at room temp. Plate was read using an ELISA reader at wave length 570. The nitrite concentration was calculated by using Na-nitrite standard curve according to **Divyagnaneswari *et al.*, (2007).**

2.6.3.2. Phagocytic activity assay: Phagocytic activity was adopted from the method described by **Wang *et al.*, (2009)** and about 200 phagocytic cells were counted and differentiated using the following equations:

$$\text{Phagocytosis \%} = \frac{\text{No of ingesting phagocytes}}{\text{Total No of phagocytes}}$$

$$\text{Phagocytic index} = \frac{\text{No of ingested Calbicans cells}}{\text{No of ingesting phagocytes}}$$

2.6.3.3. Lysozyme activity:

Serum samples were measured using the turbidometric method as described by **Esteban *et al.*, (2001)** A 25µl of serum was added onto 175 µl (0.75 mg/ml *Micrococcus lysodeikticus*) together with the assay buffer in flat-bottomed 96-well plates. The reduction in absorbance at 450 nm was measured from 0 to 15 min. at 25 C° using an ELISA reader. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min^{-1} and the units of lysozyme activity were calculated using the hen egg white lysozyme standard curve.

2.7. Challenge test:

At the end of the experimental period challenges testing of different fish groups were carried out as following 20 fish from each group were challenge I/P with 0.3ml of 24 hr. old broth culture of *A. hydrophila* contained (10^8cfu) according to **Schaperclaus *et al.*,(1992).** Another 10 fish from each group were challenged I/P with 0.5ml of 24 hr. old broth culture of *Streptococcus dysgalactiae* contained ($3.7x10^7$) according to **Hussein(2002).** All experimentally infected *O. niloticus* were maintained for a week. Clinical signs were observed and mortality rates were

recorded. Blood samples were collected with or without anticoagulant from *A. hydrophila* challenged groups not *S. dysgalactiae* ,due to high mortality so there were not enough fish for blood samples.

2.8. Statistical analysis: Data were presented as means ± standard error (SE) and the significance of differences was evaluated using analysis of variance (ANOVA) and t- student test (**SPSS 14, 2006).**

Results

2.7. Growth performance: the final weight, weight gain and SGR were significant increased ($p \leq 0.05$) in group C (feed on mixture of *SP* and *NS*) followed by groups B and A in comparison with control group. Table (2).

Hematogram , Serum Biochemistry and Immunological measurments:

2.7.1. **Hematological indices:** the results of hematogram before challenge test revealed that a significant increase in RBCs and Hb of group C in compared to other three groups. There was no significant difference between group B and A . However, both groups showed significant increase than control group in RBCs. there was significant increase in WBCs in group C followed by group B and A in compared with control group. After challenge, all treated groups showed significant increase in WBCs, RBCs, PCV and Hb in compared with control group, but when compared with before challenge there was significant decrease in RBCs in group A and C, however there was significant increase in WBCs in all groups **Table (3).**

2.7.2. **Serum biochemistry:** before challenge there was significant difference between treated groups and control in T.P, globulin and A/G ratio. There was no significant difference between groups in albumin and liver enzymes. Group C showed the higher values than other treated groups. There was no significant difference before and after challenge in all groups except control group which showed significant increase in albumin, A/G ratio and liver enzymes. There were significant increases in T.P and globulin, as well as significant decreases in albumin, A/G ratio and liver enzymes when compared with control group. **Table (4). Serum electrophoresis:** the electrophoretic pattern of serum protein pointed out the presence of 8 fractions; before challenge group C provoked a significant increase in total protein and γ globulin (may represent fractions 1&2) than other groups and showed significant decrease in albumin (may represent fraction 7) than control and group A. as well as there were significant increase in γ2and α1 globulin (may represent fractions 2&6) in other treated groups than control. Groups B and C showed significant decrease than group A in albumin (may represent fraction 7). After challenge there were significant increase in total protein and globulin (may represent fractions1-6) in treated groups in compared with control, group B showed significant decrease in albumin (may represent fraction 7) than other groups. There was no significant change in total protein before and after challenge but treated groups showed significant increase after challenge in globulin fractions especially γ globulin (may represent fraction 1 &2). **Table (5).**

2.1.1. Immunological parameters: the results of some immunological parameters of fish groups represented in **Table (6) Plate (1)** revealed that before challenge Group C showed high value than other treated groups in nitric oxide (than group A&B) and in phagocytic index & lysozyme activity (than group B). After challenge all treated group showed significant increase when compared with control group. and group

C showed significant increase than other treated groups, but when compare groups before and after challenge we found that there were significant increase in all groups after challenge in nitric oxide and lysozyme activity but in phagocytic index group C the only group showed significant increase after challenge and it was the highest value in all immunological parameter which done.

Table (2). Growth performance of different *O.niloticus* groups at the end of experimental period.

Parameters	Fish groups			
	Control	(group A)	(group B)	(group C)
Initial weight	54.4±5.97	55.4±4.52	55.2±2.17	55.4±2.35
Final weight	79.6±4.65^{A}	98.4±4.60^{a}	100.0±2.10^{a}	107.2±2.01^{a}
Weight gain	25.21±1.56^{A}	44.00±1.42^{aB}	45.2±1.21^{aC}	52.4±0.54^{abc}
SGR	0.52±0.12^{A}	0.92±0.04^{aB}	0.98±0.02^{aC}	1.18±0.04^{abc}

Data represented as means ± SE, n= 10 small letters a, b and c means significant difference against capital letters A, B and C respectively for the same item in the same column by LSD using ANOVA test at ($p \leq 0.05$).

Table (3) Hematological indices of different *O.niloticus* groups

Items		Control	Group A	Group B	Group C
RBCs x10^{6}	before	1.75±0.02^{A}	1.97±0.06^{aB}	1.98±0.05^{aC}	2.15±0.05^{abc}
	after	1.42±0.05^{A**}	1.69±0.06^{aB**}	1.86±0.03^{ab}	1.97±0.02^{ab**}
Haemoglobin (g/dl)	before	8.20±0.10^{A}	8.76±0.15^{aB}	8.62±0.16^{aC}	9.54±0.12^{abc}
	After	7.21±0.26^{A**}	8.20±0.20^{a}	8.40±0.20^{a}	8.91±0.45^{a}
PCV %	before	25.00±1.18	24.60±1.20	25.60±0.74	26.20±0.58
	after	20.00±0.60^{A**}	23.66±1.31	24.33±1.20^{a}	25.30±0.81^{a}
WBCs x10^{3}	before	33.10±1.52^{A}	36.80±0.84^{aB}	40.80±0.92^{abC}	45.00±0.72^{abc}
	after	43.00±0.50^{A**}	49.30±1.01^{a**}	49.66±0.88^{a**}	51.30±1.96^{a**}

Data represented as means ± SE, n= 5 small letters a, b and c means significant difference against capital letters A, B and C respectively for the same item in the same row by LSD using ANOVA test at ($p \leq 0.05$). * Significant difference between groups in the same Colum using t-student test at $p \leq 0.05$. **Significant difference between groups in the same Colum using t-student test at $p \leq 0.01$.

- Before mean at the end of experimental period and before challenge with *A.hydrophila*
- After mean after challenge test

Table (4) Biochemica.l analysis of different *O.niloticus* groups

Items		Control	Group A	Group B	Group C
Total protein	before	2.95±0.29^{A}	3.90±0.21^{a}	3.54±0.12^{aB}	4.32±0.14^{ab}
	after	2.59±0.13^{A}	3.73±0.04^{aB}	3.40±0.03^{abC}	3.99±0.10^{abc}
Albumin	before	1.44±0.08	1.61±0.07	1.49±0.12	1.45±0.05
	after	1.80±0.06^{A*}	1.70±0.02^{aB}	1.60±0.03^{ab}	1.68±0.01^{a}
globulin	before	1.41±0.12^{A}	2.30±0.10^{aB}	2.14±0.19^{aC}	2.87±0.15^{abc}
	after	0.86±0.13^{A*}	1.98±0.16^{a}	1.80±0.01^{aB}	2.31±0.20^{ab}
A/G ratio	before	1.02±0.06^{A}	0.70±0.05^{aB}	0.73±0.12^{aC}	0.47±0.02^{abc}
	after	2.00±0.02^{A**}	0.93±0.14^{a}	0.89±0.04^{a}	0.72±0.08^{a}
ALT	before	10.00±1.73	9.30±0.88	9.00±0.52	8.90±0.57
	after	16.00±0.57^{A**}	11.00±0.57^{a}	12.00±1.15^{a*}	11.20±0.90^{a}
AST	before	15.33±0.41	14.5±0.52	14.00±0.33	14.00±0.36
	after	19.00±0.46^{A**}	16.00±0.43^{a*}	17.20±0.51^{a**}	16.50±0.82^{a}

Data represented as means ± SE: n= 5 small letters a, b and c means significant difference against capital letters A, B and C respectively for the same item in the same row by LSD using ANOVA test at ($p \leq 0.05$). * Significant difference between groups in the same Colum using t-student test at $p \leq 0.01$. 0.05. **Significant difference between groups in the same Colum using t-student test at $p \leq 0.01$.

- Before mean at the end of experimental period and before challenge with *A.hydrophila*
- After mean after challenge test

Table (5): percentage of different serum total protein fractions (g/dl) of different *O. niloticus* groups.

Items		Control	Group A	Group B	Group C
Total protein	before	2.95 ± 0.29^{A}	3.90 ± 0.21^{a}	3.54 ± 0.12^{aB}	4.32 ± 0.14^{ab}
	after	2.59 ± 0.13^{A}	3.73 ± 0.04^{aB}	3.40 ± 0.03^{abC}	3.99 ± 0.10^{abc}
1	before	0.11 ± 0.03^{A}	0.20 ± 0.02^{B}	0.19 ± 0.01^{C}	0.32 ± 0.05^{abc}
	after	0.13 ± 0.04^{A}	$0.28\pm0.01^{a**}$	$0.26\pm0.02^{a**}$	0.33 ± 0.05^{a}
2	before	0.39 ± 0.02^{A}	0.54 ± 0.06^{aB}	0.51 ± 0.03^{aC}	1.09 ± 0.04^{abc}
	after	$0.25\pm0.04^{A**}$	$0.94\pm0.02^{a**}$	$0.92\pm0.03^{a**}$	$0.96\pm0.01^{a**}$
3	before	0.21 ± 0.01	0.44 ± 0.11	0.26 ± 0.10	0.33 ± 0.01
	after	$0.13\pm0.02^{A**}$	0.36 ± 0.01^{a}	0.39 ± 0.01^{aB}	0.31 ± 0.05^{ab}
4	before	0.22 ± 0.03	0.40 ± 0.04	0.44 ± 0.17	0.41 ± 0.03
	after	0.15 ± 0.10	0.43 ± 0.01	0.24 ± 0.09	0.36 ± 0.05
5	before	0.17 ± 0.01	0.27 ± 0.07	0.44 ± 0.02	0.34 ± 0.12
	after	0.19 ± 0.04^{A}	0.27 ± 0.05^{B}	$0.09\pm0.04^{abC**}$	0.29 ± 0.03^{ac}
6	Before	0.28 ± 0.14^{A}	0.48 ± 0.07	0.79 ± 0.10^{a}	0.76 ± 0.19^{a}
	After	0.22 ± 0.08^{A}	0.44 ± 0.01^{aB}	$0.26\pm0.06^{bC**}$	0.43 ± 0.06^{ac}
7	Before	1.42 ± 0.03^{A}	1.46 ± 0.04^{B}	0.70 ± 0.01^{ab}	0.78 ± 0.25^{ab}
	After	1.45 ± 0.11^{A}	$0.91\pm0.02^{aB**}$	$1.16\pm0.10^{b**}$	1.22 ± 0.02^{b}
8	Before	0.15 ± 0.03	0.16 ± 0.02	0.12 ± 0.07	0.14 ± 0.05
	after	$0.07\pm0.02^{*}$	0.10 ± 0.06	0.08 ± 0.03	0.09 ± 0.04

Data represented as means ± SE, n= 5 small letters a, b and c means significant difference against capital letters A, B and C respectively for the same item in the same row by LSD using ANOVA test at ($p\leq 0.05$). * Significant difference between groups in the same Colum using t-student test at $p\leq 0.05$. **Significant difference between groups in the same Colum using t-student test at $p\leq 0.01$.

- Before mean at the end of experimental period and before challenge with A. hydrophila
- After mean after challenge test
- (1-8) means protein fractions

Table (6): Immunological parameters of different O. niloticus groups.

Items		Control	Group A	Group B	Group C
Nitric oxide	before	31.01 ± 0.77^{A}	36.18 ± 2.30^{B}	35.84 ± 1.25^{C}	43.81 ± 2.78^{abc}
	after	$48.88\pm0.52^{A**}$	$51.00\pm0.84^{aB**}$	$51.10\pm0.87^{aC**}$	$53.67\pm0.59^{abc**}$
Phagocytic index	before	1.45 ± 0.12^{A}	1.84 ± 0.09^{a}	1.60 ± 0.15^{B}	2.00 ± 0.13^{ab}
	after	1.52 ± 0.15^{A}	2.00 ± 0.10^{aB}	1.86 ± 0.05^{aC}	$2.50\pm0.09^{abc**}$
Lysozyme activity	before	16.85 ± 1.90^{A}	22.72 ± 1.82^{a}	25.88 ± 1.85^{aB}	21.86 ± 0.77^{ab}
	after	$22.72\pm0.38^{A*}$	$30.94\pm1.89^{aB*}$	$40.42\pm1.99^{abC**}$	$45.61\pm2.00^{abc**}$

Data represented as means ± SE, n= 5 small letters a, b and c means significant difference against capital letters A, B and C respectively for the same item in the same row by LSD using ANOVA test at ($p\leq 0.05$). * Significant difference between groups in the same Colum using t-student test at $p\leq 0.05$. **Significant difference between groups in the same Colum using t-student test at $p\leq 0.01$.

- Before mean at the end of experimental period and before challenge with A. hydrophila
- After mean after challenge test

Table (7) Challenge tests of *O. niloticus* groups

Items	Mortality %				
	Control negative %	Control positive %	Group A %	Group B %	Group C %
I/P. injection of (0.3ml contain 10^{8}cfu/ml) A. hydrophila	0	70	50	45	30
I/P. injection of (0.5ml contain $3.7x10^{7}$ cfu/ml) Streptococcus dysgalactiae	0	80	50	50	40

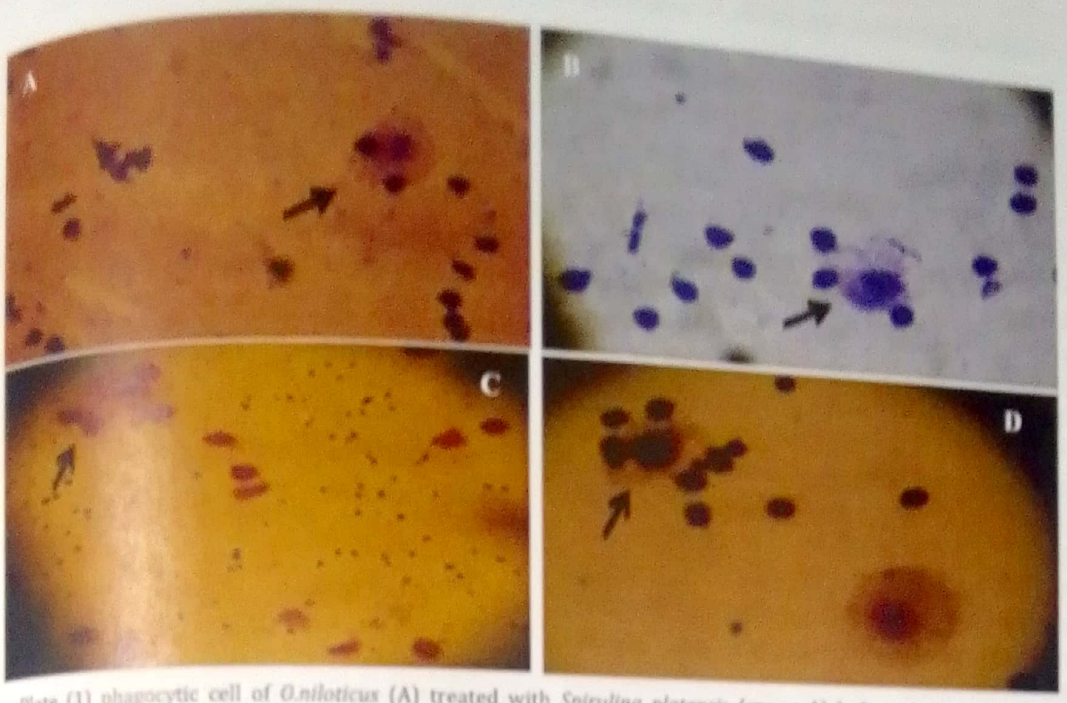

Plate (1) phagocytic cell of *O.niloticus* (A) treated with *Spirulina platensis* (group A) before challenged with *A.hydrophila* engulfed more than one C. albicns (B) treated with combination of *Spirulina platensis* and *Nigella sativa* (group C) before challenged with *A.hydrophila* engulfed more than one C. albicns (C) treated with *Nigella sativa* (group B) after challenged with *A.hydrophila* engulfed more than one C. albicns (D)group C after challenged with *A.hydrophila* engulfed more than one C. albicns (Giemsa stain X-1000)

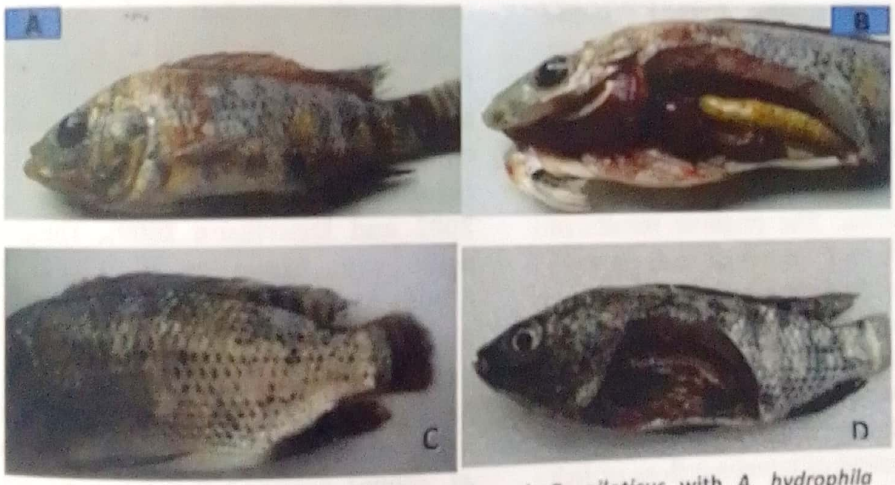

Plate (2) showing: (A) experimentally infected *O. niloticus* with *A. hydrophila* showing external hemorrhage, detached scales and internally (B) showing congestion of internal organ. (C) Experimentally infected *O. niloticus* with *S. dysgalactiae* showing tail rot and detached scales and (D) showing dark skin, Tail rot and congestion of internal organs.

3.3*Challenge test:* The results of challenge tests with the pathogenic strains of *Aeromonas hydrophila* and *Streptococcus dysgalactiae* indicated that, the mortality percentages of treated fish groups were significantly decreased versus positive control group. The control group has demonstrated the highest mortality percent. However, a group C (which treated with mixture of *Spirulina* and *Nigella sativa*) showed the lowest mortality percent between other treated groups. **Table (7),**

between other treated groups. **Table (7),** clinical signs and PM lesions were shown in **Plate (2).**

Discussion

The use of immunostimulants as an alternative to the drugs, chemical and antibiotics currently is being used to control fish diseases in fish culture **(Mukesh *et al.*, 2012).** Immunostimulants increase resistance to infectious diseases not by enhancing specific immune responses but by enhancing non-specific defense mechanisms.The present study was performed to evaluate the effect of both *SP* and *NS* or combination between them as feed additives as immunostimulants for control of some bacterial diseases.

Concerning the statistical analysis of different growth parameters in *O.niloticus* fed on diet supplemented with *Spirulina platensis*, *Nigella sativa* and combination revealed that there were significant increases in final body weight, weight gain and SGR in all treated groups than control group also group C showed the highest value of weight gain and SGR than other treated groups, Suggesting that the addition of *SP*, *NS and* combination enhance the growth performance and mitigated the effects of population density in glass aquaria which is the main growth inhibiting factor in intensive aquaculture system. The positive results of *Spirulina* additive (group A) on growth performance of *O. niloticus* as indicated by significant increase in body weight, weight gain and SGR were similar to the results obtained by **Sherif *et al.*,(2012) Fadi *et al.*, (2013) and Mai *et al.*,(2013)** and may be due to improvement of intestinal flora of fish rendering breakdown of indigestible feed component to extract more nutrients from the feed. This also stimulates the production of enzymes that transport fats within the fish for metabolism instead of storage as suggested by **James *et al.*, (2006),** or due to high protein content and unsaturated fatty acid which found in *Spirulina* **(Reitan *et al.*, 1993).** Also, positive results of *Nigella sativa* (group B) agree with **Walaa, (2008) and Abd el Wahab, (2012)** were reported that *NS* improved growth performance in *O. niloticus* and these results may be due to *NS* contains a lot of valuable components. It is a significant source of protein , essential fatty acids and many vitamins such as vitamin A which play an important role in growth and maturation of the cells, vitamin B1, B2, B3,which is important for release of energy from carbohydrate and fats , and vitamin C which play an important role in immunity **(Gibson and Roberfroid, 1995).** The highest value of group C in weight gain and SGR could be justified to the synergistic interaction of *Spirulina platensis* and *Nigella sativa* and lead us to believe that use of mixture can be more stimulatory than individual or single compound.

The hematological parameters are an important tool of diagnosis that reveals the state of health of fish **(Rehulka, 2002).** Also explains abnormalities caused by immunostimulants. The results of haemogram in this study at the end of experimental period indicated that there were significant increases in RBCs, WBCs and Hb in all treated groups in compared with control group. Group C showed the highest value between treated groups, these results may be due to that both *Spirulina* and *Nigella sativa* considered to be rich sources for iron , vitamin B12, B1,B6 and minerals also *Spirulina* contain phycocyanin, and chlorophyll which have antioxidant effect **(Mosulishvili 2002; Muhammad *et al.*, 2002 and Henrikson 2009).** These results agree with **Mona *et al.*, (2002) Abeer, (2005) Walaa, (2008) Fadi *et al.*, (2013) and Mai *et al.*, (2013)** who reported that both of *Spirulina Platensis* and *Nigella sativa* extract has positive effect on hematological parameter also **Watanuki *et al.* (2006)** reported that Spirulina activated the functions of leucocytes in common carp, *Cyprinus carpio*.

The results after challenge showed that although there was significant decrease in RBCs in some groups as A and C, there was no significant change in Hb except control group showed significant decrease in RBCs, Hb and PCV, these results may be due to toxin secreted by bacteria as hemolysin which cause hemorrhage. Also, **Kumar and Ramulu (2013)** reported that *A. hydrophila* cause hypochromic microcytic anemia. The treated groups showed no

change in Hb this may be due to that treated groups can combat the bacterial infection, this appeared from the significant increase in WBCs. Group C showed the highest value in WBCs meanwhile the effect of *A. hydrophila* on treated groups especially group C was mild in compared with control group, the data support the present finding that the mortality rate of treated groups was less than control group These results were more or less agree with **Zaki *et al.*,(2011)** who reported that *Nigella sativa* and Ginseg improved hematological parameters of catfish infected by aflatoxin, and **Neveen and Ibraheem, (2008)** said that *Spirulina* has inhibitory substances inhibited four species of *A. hydrophila*.

Generally, increases in the levels of serum protein, albumin and globulin in fish are thought to be associated with a stronger innate response **(Wiegertjes *et al.*, 1996).** The measurement of albumin, globulin, and total protein in serum or plasma is 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 **(Schaperclaus *et al.*, 1992).** Many authors reported the positive effect of *Spirulina* on total protein and its fractions as **Abdel-Tawwab *et al.*,(2008) Sherif *et al.*, (2012) and Fadi *et al.*, (2013).** Other authors report the positive effect of *Nigella sativa* **Diab *et al.*, 2008; Walaa, 2008** and **Elkamel and Mosaad 2012).** In the present study, total protein and globulin of all treated groups showed significant increases in compared with control. However group C the highest value between treated groups. There was no significant change in albumin in addition to significant decrease in A/G ratio in all treated groups especially group C. These results mean that the used immunostimulants enhanced immunity and improve *O. niloticus* health. These results were not change after *A. hydrophila* challenge except control group which showed significant increase of albumin and A/G ratio and significant decrease in globulin. These may due to the effect of bacterial toxins and this mean the good health status of treated groups to overcome stress effect of infection which lead to decrease total protein as under stress conditions. The protein consumed by fishes is not stored in the body tissue **(Baskaran & Palanichamy, 1990)** and hence, the stressed fish meet their extra energy requirements from body proteins, which are mobilized to produce glucose, that is made available for fishes by the process of gluconeogenesis. So, this depletion of the protein levels may have been due to its utilization for metabolic purposes.

Globulin is made up of fractions of α1, α2, β, and γ globulins, which are considered as the source of almost all the immunologically active protein in the blood **(Jha *et al.*, 2007).** In the present study, all treated groups showed significant increase in α1 and γ globulins in compare with control group and group C (treated with *SP* and *NS*) showed the highest value of γ globulin between treated groups which mean activation of humeral immune system. After challenge there were significant increases in globulin fraction in treated group especially γ globulin These results indicated the role of *Nigella sativa* and *Spirulina platensis* as immunostimulants not only on the non-specific immune response, but also it may enhance some specific body defense.

The measurement of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are considerable diagnostic value in fish as it relate to general nutritional status as well as liver function **(Schaperclaus *et al.*, 1992).** In this study there were no significant differences in level of serum ALT and AST between all groups so *SP* and *NS* showed no adverse effects on liver function and have good nutritional status in addition to integrity of vascular system these results agree with **Sherif *et al.*, (2012).** After challenge, there were significant increases in ALT and AST of control group and mild increase in AST in treated groups when compared with the results before challenge and control. These results are agree with **Grizzle and kiryu , (1993)** which stated that serum level of AST increase after experimental or natural infection by Aeromonas spp .

Phagocytic cells (monocytes, neutrophils, macrophages and dendritic cells)

play very important roles in the host during the early period of infection. Phagocytes survey the host for antigens, destroying them through a process known as phagocytosis (**Wiley *et al.*, 2011**) . Phagocytosis plays an important role in antibacterial defenses in teleost fish, the nitric oxide one of the most destructive products produced by activated macrophages. Increase of reactive nitrogen can be correlated with increase of oxygen and nitric oxide radicals production and increase of killing activity (**Sharp and Secombes, 1993**). Many reports described that *Spirulina* and *Nigella sativa* showed significant increase phagocytic activity and nitric oxide in *O. niloticus* fish (**Tayag, 2010; Elkamel and Mosaad, 2012; Hany *et al.*, 2012; Sherif *et al.*, 2012**). The same results were recorded in this study but group C showed the highest value of phagocytic percentage, phagocytic index and nitric oxide between all groups. The results after challenge with *A. hydrophila* showed the significant increase in all treated groups than control in phagocytic percentage and nitric oxide but group C the only group showed significant increases in phagocytic index, this means that combination of *Spirulina* and *Nigella Sativa* increase the activity of macrophages to engulf large number of bacteria these results agree with the results of challenge test in this study which revealed that group C the lowest mortality rate in *A. hydrophila* and *S. dysgalactiae*. Lysozyme is a lytic enzyme that plays an important role in preventing the invasion of microbes by splitting the β (1-4) linkages between N-acetylmuramic acid and acetylglucosamine of bacterial cells thus resulting in lysis (**Galindo-Villegas & Hosokawa, 2004**). Also, lysozyme exerts a role in activating phagocytes and the complement system (**Grinde, 1989**). The results of this study revealed significant increase in lysozyme activity in all treated groups, the results were in accordance with **Tayag *et al.*, (2010) and Mai *et al.*, (2013)** who reported that *Spirulina* increase lysozyme activity and **Alishahi *et al.*, (2012) and Awad *et al.*, (2013)** who reported that *Nigella sativa* showed the highest level of lysozyme at 0.5 and 3% respectively. After challenge, group C showed the highest level of lysozyme mixed [illegible] highly lysozyme level which is plays an important role in the host defense mechanisms against the two bacterial pathogen .

The disease challenge is an in vitro technique provides an opportunity to determine the performance and immunity of the fish species ebon exposure to bacteria on their natural habitats (**AraKoosh *et al.*, 2009**). Concerning the challenge tests applied in the fish groups with gram positive and gram negative bacteria the first test was performed by IP injection with virulent strain of *A. hydrophila*. The results indicated the appearance of characteristic clinical signs in ***O. niloticus*** control group as early as three days post challenge with a total mortality percentage of 70%. These clinical signs were attributed to the effect of a varity of virulence factors such as extracelluler products (haemolysine , proteases and acetylcholine esterase) which attack endothelial lining of blood vessels and parenchymatous organ causing haemorrhgic phenomena and such bacteria were transmitted from fish to other by direct contact or through water (**Nieto *et al.*, *1991*and Angka *et al., 1995*).** On the other hand, ***O. niloticus*** in groups kept on diet supplemented with *Nigella sativa*, *Spirulina platensis* and combination between them showed lower mortality percentage in group C (30%) followed by groups B and A(45and50), these results supported by the finding of **Walaa, (2008)** who reported that *Nigella sativa* reduced the mortality rate than control. Also, **Sherif *et al.*, (2012)** reported that *O. niloticus* feed on *Spirulina platensis* recorded lower mortality rates (ranged between 33.3 and 40%) compared to control

In case of challenge with *S. dysgalactiae* group C, also showed the lower mortality rate (40%) followed by group A and B (50%) in compared with control group. The lower rate of mortality either in *A. hydrophila* or *S. dysgalactiae* as a result of good health condition as well as good immune status represented in high level of hematological and immunological parameters, the results of challenge supported the results of in vitro antibacterial activity of either *Nigella sativa* or *Spirulina*

platensis in this study , and support other research which revealed that *Nigella sativa* contain active material known as nigellon, thymoquinone and thymohydroquinone that appear to possess antibacterial effect **(Hanafy and Hatem 1991)**. *Spirulina platensis* contain C- phycocyanin which has antibacterial effect (**Sarada *et al.*, 2011**).

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الملخص العربى

اجريت هذة الدراسة لتقييم تأثير كلا من السبيريولينا او حبة البركة او كلاهما بجرعات 7.5 جرام/ كيلو جرام علف سبيريولينا و 0.5 جرام /100 جرام علف حبة البركة او كلاهما كاضافات اعلاف لمدة 8 اسابيع على معدلات النمو وصورة الدم والحالة المناعية قبل وبعد العدوى البكتيريا بميكروب الايروموناس هيدروفيلا فى اسماك البلطى النيلى وايضا الحماية ضد العدوى البكتيريا لميكروب السبحى المكور نوع ديس جالاكتيا وايضا ميكروب الايروموناس هيدروفيلا. وقد اسفرت النتائج عن ان اضافة السبيريولينا او حبة البركة للاعلاف ادى الى تحسن معدلات النمو والحالة الصحية وايضا تحسن فى مناعة الاسماك متمثلا فى زيادة فى نشاط الخلايا الاكولة وزيادة فى انتاج الانزيمات المحللة (ليسوزيم) و المركبات النيتروجينية و نسبة البروتين الكلى والجلوبيولين وخاصة الجاما جلوبيولين سواء قبل او بعد العدوى البكتيرية. وقد اوضحت النتائج ان المجموعة المعالجة بالسبيريولينا وحبة البركة معا اظهرت اعلى معدلات نمو واعلى كفاءة مناعية واقل نسبة نفوق عند العدوى البكتيرية)