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The Role of Nigella sativa in improving the immune response of the African Catfish *(Clarias gariepinus)* to *Aeromonas hydrophila* Vaccine

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ABSTRACT

In the past few decades, the search for alternative strategies for disease management other than chemotherapeutants has been encouraged. Recently, disease prevention among fishes depends mainly on the use of vaccine and immunostimulants together with optimal husbandry condition. The present study examined the effects of Nigella sativa (NS) on the response of catfish (*Clarias gariepinus* to vaccination and/or challenge infection. For this purpose, four hundred catfish allocated into four groups with two feeding regimes (basal diet (G1 & G2) and diet supplemented with *NS* at dose level of 3 g/100 g diet (G3 & G4). The groups (G2 & G4) are intraperitoneally (IP) vaccinated with 0.1 ml formalin-killed *Aeromonas hydrophila* bacterin. The effects of NS in G3 and G4 are evaluated in comparison with other groups (G1 & G2) using hematological and biochemical parameters together with histopathological examinations. Six weeks after the start of experiment, the results revealed significant increase in erythrogram (RBCs, Hb and PCV%), Leucogram (total Differential leucocytic count), serum total protein and globulin in catfish supplemented with 3% *NS*. On the other hand, no significant changes in liver enzymes, urea, uric acid and creatinine among all experimental groups were noticed. The histopathology revealed proliferation of hematopoietic tissues and activation of melanomacrophages in both supplemented and vaccinated groups. Two weeks after challenge experiment, vaccinated groups (G2 and G4) showed significant increase in the antibody titer while treated groups supplemented with 3% NS showed significant increase in the phagocytic activity. After challenge, catfish vaccinated and supplemented with 3% NS showed highest (95%)relative level of protection (RLP) followed by vaccinated group (85%) then supplemented group(55%). *Nigella sativa* (3 g/100 g diet) enhance the immune response and resistance of catfish to *A. hydrophila* and consequently has a potential value for aquaculture.

INTRODUCTION

During last decades, fish farming have been plagued with bacterial disease problems that limiting the expansion of aquaculture and have a significant effect on the economic development in Egypt (FAO, 2012). Epizootics of *Aeromonas*

hydrophila are troublesome in open pond culture and under intensive culture techniques, particularly with poor environmental conditions worldwide including Egypt (Aly *et al*., 1998 and Zhang *et al*., 2014). *A. hydrophila* is considered as opportunistic and ubiquitous bacteria induce "Motile Aeromonas Septicemia" (MAS), "Hemorrhagic Septicemia" "Ulcer Disease" or "Red-Sore Disease." (Swann and White, 1989 and Roberts *et al*., 1992).

Strategies for combating diseases induced by *A. hydrophila* include antibiotic application. Although antibiotics have positive effects in the control of the disease but its use become limited due to negative impact effects. These effects are consequence to the development of antibiotic resistance in bacteria and cross-resistance against human antimicrobials, suppression of immunity in host organisms and residues in fish products and in the environment (Flores *et al*., 2003, Cabello, 2006, Romero *et al*., 2012 and Aly and Albutti, 2014). Consequently, finding favorable alternatives strategies for controlling bacterial pathogens in aquaculture was encouraged by Health Organizations. In aquaculture industry, fish vaccination has been proved very important in preventing infectious diseases and reducing the losses induced by the diseases (Ellis *et al*., 1997). Several kinds of vaccines, including whole cell (WC), Outer Membrane Protein (OMPs), extra cellular products (ECPs), lipopolysaccharide (LPS) and biofilms, confirmed to overcome the disease caused by *A. hydrophila*. The success of vaccination programs for controlling *A. hydrophila* mediated disease problems is difficult due to its various heterogeneity and stereotyping (Badran *et al*., 1993). In the past decade, another alternative protocol to prevent the diseases through strengthening the innate immuneresponses of fish without prior exposure to an antigen (Galindo-Villegas and Hosokawa, 2014). This protocol is called immun-ostimulants that often act through targeting complement activation, phagocytosis and cytokines secretion.

Several compounds from synthetic origin are tested in fish as effective immunostimulants used as adjuvants for vaccination process as mineral oil and Freund's complete adjuvants (Poppe and Breck, 1997 and Chillingworth and Donaldson, 2003). However, the undesirable side effects reported and expressed physiologically and morphologically for synthetic immunostimulants limited its use in aquaculture (Skinner *et al*., 2008, Aunsmo *et al*., 2009 and Bjerkas *et al*., 2010).

Nowadays, immunostimulants from natural origin are much appreciated and confirmed for several plants (Tafalla *et al*., 2013). Arab people have great confidence of the advantage of *Nigella sativa*as healing remedies for many ailments and have different names as Habbat Albarakah, Alhabahat Alsawda and Alkamoun Alaswad (Aljawezjjah, 2001). In other countries, it is called as Shuniz and Khodhira and in English called Black Cumin or Black Caraway (El-Tahir *et al*., 2006). It has been used for a various health troubles related to respiratory health, gastrointestinal health, renal and hepatic function, circulatory and immune system support and general well-being (Khan *et al*., 2003). Additionally, several biological effects have been proved to *N. sativa* or its active ingredients, thymoquinone and nigellone, as anti-nociceptive, anti–inflammatory, anti-cancer (Abdel-Fattah *et al*., 2000, Mbarek *et al*., 2007, Banerjee *et al*., 2010), antibacterial, fungicidal effects (Mashhadan and Rakhshandeh 2005 and Khan *et al*., 2003) . Moreover, Immunomodulatory and positive effects on the immune system of some fish species have been demonstrated for *N. sativa* (Abdel-Ghaffar *et al*., 2003, Salem, 2005 and Diab *et al*., 2008).

The present study aimed to investigate the role of *N. sativa* as immunostimulant, disease protectant and vaccine enhancer against *A.hydrophila* infection in catfish,

Clarias gariepinus through hematological, biochemical and immunological indices as well as histopathological studies.

MATERIALS AND METHODS

Fish:

Four hundred apparently healthy catfish, *Clarias gariepinus* with an average body weight of 150 + 15g were obtained from Lake Temsah at Ismailia, Egypt and transported to the Aquatic Animals Lab., Faculty of Veterinary Medicine, Suez Canal University, Egypt where they kept in well prepared aquaria. They were divided into four equal groups (each group of 4 replicates, 25 each) and each group was equally reared in 4 glass aquaria (50 × 60 × 70 cm). The aquaria were filled with freshwater that renewed 20% daily. Fish were kept for 2 weeks prior to the experiment for acclimation and fed a balanced ration (Table 1) during the study. The water quality kept in the normal range throughout the experiment [NO_3 (0.20 mg/L), NH4 (0.2 mg/L), Chl at (42.27 mg/L), available P (0.02 mg/L)]. Water temperature during the experiment was optimal (22 ± 2°C) for the culture of catfish.

Table 1. Composition of the basal diet used throughout the experiment

Ingredients	Diet (%)	Protein (%)		Metabolic energy (Joules)	
		ingredients	Feed	ingredients	Feed
Fish meal	8.00	0.72	5.76	4000	32000
Soybean meal	52.9	0.48	25.392	2870	151823
Ground corn	29.1	0.109	3.1719	1240	36084
Wheat flour	5.00	0.134	0.67	2700	13500
Vegetable oil	2.00	0.00	0.00	9100	18200
Cod liver oil	2.00	0.00	0.00	9100	18200
Di calcium phosphate	1.00	0.00	0.00	0.00	0000
Mineral mix.	0.07	0.00	0.00	0.00	0000
Vitamin mix.	0.05	0.00	0.00	0.00	0000
Vitamin C	0.03	0.00	0.00	0.00	0000
Total	100.15	0.00	34.9939	0.00	269807

Ingredients were obtained from local markets.

Diet Preparation:

Diets containing 35% protein were prepared in the form of pellets as follow: Ingredients obtained were grinded to granules (0.5 mm mesh size) (Thomes-Willey Laboratory Mill Model 4, Swedesboro, NJ 08085 U.S.A) and then mixed mechanically by horizontal mixer (Hobart model D300T, U.S.A.) at a low speed for 30 min. Oil (vegetable & cod liver) was added gradually to assure the homogeneity of the ingredients.

Black seed (*Nigella sativa*) was procured from Veterinary Pharmacy in a powder form and mixed with the formulated diet at a rate of3g/100g feed. Batches of feed were prepared every two weeks and the pellets were left for 24hr for air-drying and stored in a refrigerator (4°C) for daily use.

Bacterial pathogen:

A reference strain of pathogenic *Aeromonas hydrophila* was obtained from the Dept. of Fish Diseases and Management, Fac. Vet. Medicine, Suez Canal University, Ismailia, Egypt. The isolate was used in the vaccination trial and to test response of the experimented catfish.

Vaccine preparation:

Vaccine preparation, sterility and safety test were done as described in our previous publication (Aly *et al*., 2015). Briefly, adding formalin (0.3%) to the culture

of *Aeromonas hydrophila* that incubated at 35^{o}C for 48 h. The prepared formalized bacterial culture was kept at room temperature overnight, and then tested for sterility and safety (Cardella and Eimers, 1990). The prepared and tested vaccine is stored in the refrigerator at 4^{O}C. Immediately before use, Formalin killed bacterial cells were prepared in a concentration of 3 mg wet-weight/ml saline.

Experimental design:

All fish are subjected under two feeding regimes (basal diet (G1 & G2) and supplemented diet (G3 & G4). G2 & G4 were vaccinated. The vaccination was done through intraperitoneal (IP) injection with 0.1 ml formalin-killed *A. hydrophila* bacterin after being anesthetized by immersion in water containing 0.1 ppm MS-222 (Aly *et al*., 2015 and Cardella and Eimers, 1990). The non-vaccinated groups (G1 & G3) were IP administered 0.1 ml sterile saline solution. Nigella sativa was given at dose level of 3 g/100 g diet (Table 2). Uneaten diet was removed by a siphon after 1 hr of feeding to avoid the reduced quality and opaqueness of the water. The experiment was carried out for six weeks.

Table 2: Design of experiment in African catfish.

Groups	treatment	Total No. of Fish	Feeding regime	Vaccination	
				Saline	*Aeromonas* vaccine
1	Control negative	100	Basal Diet	√	-
2	Vaccine	100	Basal Diet	-	√
3	Nigella Sativa (NS)	100	Supplemented Diet	√	-
4	NS & Vaccine	100	Supplemented Diet		√

Blood samples and analyses:

Five catfish from each group were selected and anesthetized by immersion in water containing 0.1 ppm MS-222 (Tricain methane sulfonate; Argent Chemical Laboratories, Fisheries Division). After that, blood samples were obtained from caudal vein at the end 4th and 6th week of the feeding trial. Half of the newly collected blood samples were used to determine the hematological parameters. Another half of the blood was centrifuged for 15 min at 3500 g. After centrifugation, serums were stored at -20 ºC for future analysis.

Hematological assay:

The hematological indices {Red blood cell (RBC) count (X10^{6} per □l), hemoglobin (Hb) (g/dL) and packed cell volume (%)} were designated according to the methods of (Natt and Herick, (1952), Wintrob, (1967) and Drabkin, 1949). The percentage and absolute values for different leukocytic cells were measured according to (Miller and Seward, 1971 and Schalm, 1986).

Biochemical assay and histopathological examinations:

The determination of serum levels of total proteins, aspartate aminotransferase (AST), alanine aminotransferase ALT, serum creatinine,and uric acid were done using commercially diagnostic kit (Bioanalytic Diagnostic Industry, Co.). Specific antibody titers (Baba *et al*., 1988), phagocytosis (Torky, H. A. and Diallo, 1983 and Leiboled, 1981) and histopathological techniques (Drury and Wallington, 1980) were also done**.**

Challenge test:

The artificial infection of all groups was done at the end of the 4thweeks post-vaccination by immersion of fish (40 fish/challenge) in diluted 24 hrs broth culture of virulent *A.hydrophila*. The challenged fish were kept under observation for 2 weeks and the dead ones were used for *A. hydrophila* re-isolation. The relative level of protection (RLP) among the challenged fish was determined:

RLP % = 1 - (% stimulated mortality ÷ percent mortality in control group) X100 (Ruangroupan *et al*., 1986).

Statistical analysis:

One-way and two-way analyses of variance (ANOVA) were carried out. Also, Duncan's Multiple Range Test (Dauncan, 1955) was used to determine differences among treatments (mean at significance level of P<0.05). Standard errors were also estimated. Analysis was carried out using the SAS package (SAS, 2005).

RESULTS

There were significant increase in RBCs, Hb and PCV % in G3 and G4 (p>0.05) while no significant differences among the control and vaccinated groups. The total leucocytic and lymphocytic counts showed significant increase at p>0.05in G2 and G3 and showed high significant increase at p>0.01 in G4 (Table 3).

Table 3: Erythrogram and Leucogram in African catfish at the end of 6-weeks experiment.

Groups / Parameters	G1	G2	G3	G4
RBC. (10^6/□L)	2.40 ±0.03	2.49 ±±0.15	2.80 ±0.10*	2.87±0.25 *
Hb. (g/dL)	9.48± 0.01	9.54±0.39	10.01±0.29*	11.06±0.48 *
PCV (%)	27.24± 0.02	28.45 ±0.46	28.15 ±0.11 *	29.45 ±0.79 *
T.L.C.(10^3/□L)	26.25 ±0.02	33.58±0.02*	28.99±0.03 *	37.75±0.03**
Neutrophil(10^3/□L)	9.50±0.02	10.30±0.031	10.10±0.02	10.10±0.02
Lymphocye(10^3/□L)	15.12±0.05	18.65±0.03*	17.751 ±0.04 *	25.10±0.04**
Monocyte(10^3/□L)	0.49±0.01	0.58±0.01*	0.44±0.01	0.52±0.01
Eosinophil(10^3/□L)	1.111±0.003	1.132±0.001	1.026±0.001	1.114±0.003
Basophil (10^3/□L)	0.101 ±0.004	0.102±0.002	0.105±0.004	0.103±0.002

Data are expressed as mean + S.E (n=5/group). * Significant at P<0.05, ** Highly Significant at P<0.01.

There was a significant increase in total protein in G2, G3 and G4 while globulins levels increased significantly in G3 and G4 (Tables 3). On the other hand, no significant changes in liver enzymes, urea, uric acid and creatinine among all experimental groups were recorded (Table 4). Additionally, vaccinated groups (G2 and G4) showed significant increase in the antibody titer (Table 5) while groups supplemented with NS in their diet (G3 and G4)showed significant increase in the phagocytic percentage (Table 5).

Table 4: Serum biochemical parameters of African catfish fed diets containing Nigella sativa at 6weeks of experiment.

Groups / Parameters	G1	G2	G3	G4
T.P (g/dl)	6.11±0.12	7.32±0.15*	7.56±0.18*	9.42±0.25**
Albumin (g/dl)	2.55±0.12	2.45±0.09	2.57±0.14	2.55±0.16
Globulin (g/dl)	3.21±0.10	4.52±0.13	4.75±0.12*	6.44±0.18*
A/G Ratio	0.711 ±0.09	0.452 ±0.05	0.438 ±0.06	0.384 ±0.01
AST (u/L)	22.31 ±0.24	25.31 ±0.25	22.17 ±0.16	23.51 ±0.34
ALT (u/L)	15.63 ±0.38	17.57 ±0.29	15.65 ±0.22	16.45 ±0.20
Uric acid (mg/dl)	2.48 ±0.06	2.59 ±0.09	1.99±0.07	2.16±0.04
Urea (mg/dl)	3.45±0.02	3.55±0.05	3.47±0.01	3.50±0.02
Creatinin(mg/dl)	0.57±0.03	0.58±0.03	0.52±0.04	0.53±0.01

Data were expressed as mean ± SE (n=5/group).

* Significant at P<0.05, ** Highly Significant at P<0.01.

Table 5: Antibody titer, phagocytic percentage, mortalities percentage and relative level of protection in African catfish challenged with *A. hydrophila*.

Group	Period (week)	Antibody titers	Phagocytic %	Mortalities %	RLP %
G1	4th	2.10 ± 0.02	41.91 ± 1.07	85.00-	-
(control)	6th	2.10 ± 0.02	41.37 ± 0.92		-
G2	4th	8.33 ± 0.03* *	47.82 ± 1.03	12.50-	85.29.-
(vaccinated)	6th	6.52 ± 0.06*	41.67 ± 0.92		
G3	4th	2.15 ± 0.03	48.97 ± 1.03*	37.5-	55.88-
(Treated)	6th	2.21 ± 0.12	45.26 ± 1.07		
G4	4th	9.99 ± 0.02 *	75.15 ±1.04**	5-	94.11-
(Treated & Vaccinated)	6th	7.58 ± 0.01*	65.58 ± 1.07*		

Data were expressed as mean ± SE (n=5/group).
* Significant at P<0.05, ** Highly Significant at P<0.01.

The highest protection after challenge infection with *A. hydrophila* was recorded in fish in G2 (vaccinated only) and G4 (vaccinated & supplemented with NS at 3%) accounted to 85 and 95% followed by G3 (fish supplemented with diet containing NS at 3%). The mortality percentage was the highest (85%) in the control group and the lowest (5%) in G4 (Table 5).

Histopathology, Fish of control group showed normal microscopic picture of all examined organs with no marked changes in the hematopoietic organs along the period of experiment. Vaccinated group showed mild cellular degeneration along with moderate proliferation of the hematopoietic tissue and melanomacrophages cells in the parenchymatous organs (Fig. 1).

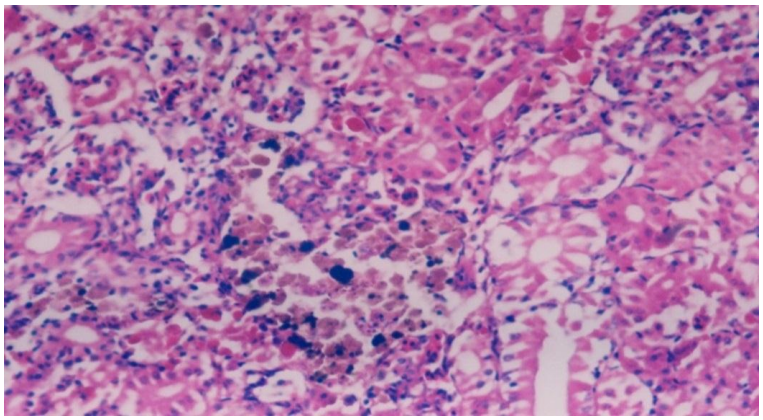

Fig. 1: Kidney, Catfish of Vaccinated group, showing tubular nephrosis with moderate proliferation of the hematopoietic tissue and melanomacrophages cells.*H&E, X250.*

Catfish of group 3 supplemented with NS diet showed an excessive proliferation of the renal hematopoietic tissue with mild tubular degeneration while the liver suffered mild degenerative changes (vacuolar degeneration) of hepatocytes together with proliferation of melanomacrophages cells (Fig. 2). The spleen revealed activation of melanomacrophages and proliferation of lymphoid cells. Catfish of G4 (supplemented with NS & vaccinated) showed excessive proliferation of the hematopoietic tissue together with marked activation of melanomacrophages cells in the parenchymatous organs (Fig. 3).

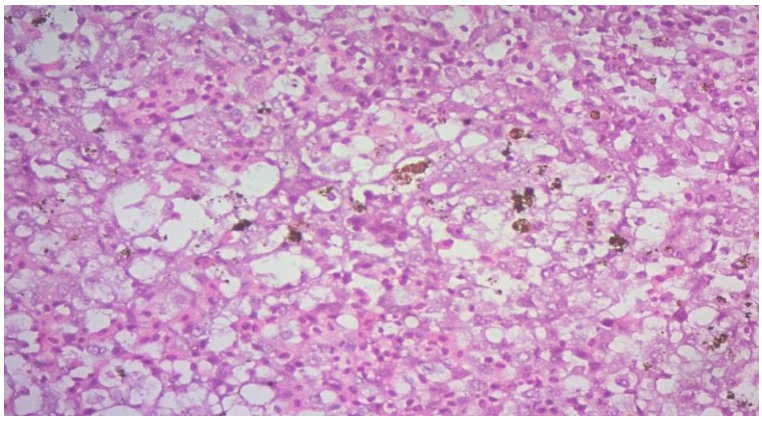

Fig. 2: Liver, Catfish supplemented with NS, showing vacuolar degeneration of hepatocytes together with proliferation of melanomacrophages cells.*H&E, X250.*

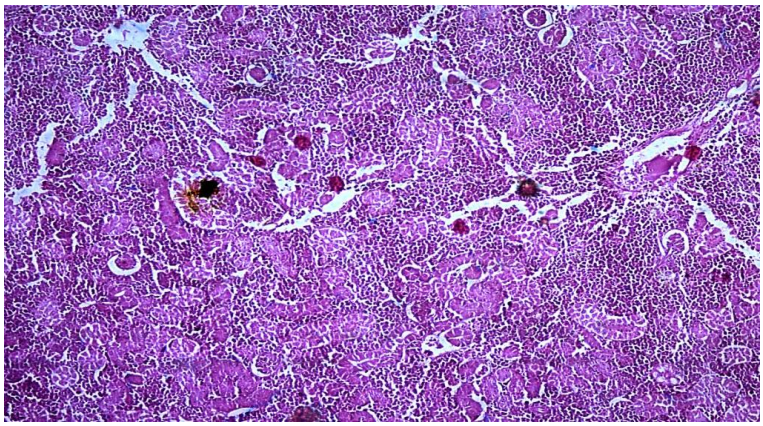

Fig. 3: Kidney, Catfish of vaccinated & supplemented group, showing excessive proliferation of the hematopoietic tissue with marked activation of melanomacrophages cells.*H&E, X100.*

DISCUSSION

The use of antibiotics as growth promoters or disease protectants in aquaculture was limited due to negative impact effects that may pose hazards on human health (Romero *et al*., 2012). Components from natural plant origin are demonstrated as best alternatives for these chemicals (Tafalla *et al*., 2013). The present study revealed that, diet supplemented with NS (3%) gave promising results in the field of immunostimulants in aquaculture. Blood in fish reflect the pathological and physiological health condition of the body. In addition, changes in blood components are recorded during the use of immunostimulants (Tewary and Patra, 2011). The present study revealed significant increase in complete blood picture (erythrogram and leucogram except neutrophil) in catfish supplemented with NS. Although, the result is inconsistent with that previously reported (Abdelwahab and El-Bahr, 2012) in Rainbow trout, it is consistent with other studies tested NS either in catfish (Abeer, 2005) or other fish as Nile tilapia (Hussein, 2000 and Elkamel and Mosaad, 2012) and Sea-bass (Saad *et al*., 2013). The activation of lymphoid and hemopiotic tissues that recognized in the current study during the histopathological examinations may explain such increase happed after NS supplementation as suggested by Satish *et al*., 1991). Additionally, Hb percentage increase may be due to activation of enzymes contribute in Hb synthesis (El-Tahir *et al*., 1993) or increasing the size of red blood cells (El-Feki *et al*., 1993) that resulted from the activation of the hematopoietic tissues that observed microscopically in the treated catfish.

Serum protein and immunoglobulin are an important part of humoral immune system of vertebrates (Salem, 2005).The increase in the levels of serum total protein and globulins in catfish recorded in groups supplemented with NS was in agreement with that recorded in catfish (Abeer, 2005); Sea-bass (Saad *et al*., 2013) and rainbow trout (Dorucu *et al*., 2009 and Awad *et al*., 2013). Such increase in protein pattern could be partly due to NS high content of crude protein (20.5%) and free amino acids (Babayan *et al*., 1978, Hedaya, 1995 and Atta. 2003) and partly due to enhancement of innate immunity response (Wiegertjes *et al*., 1996). The significant increase in serum globulins indicated the immunostimulant effect of NS as previously reported (Aqel, 1993 and Awad *et al*., 2013). Moreover, the recent findings where NS induced significant increase in alpha, beta and gamma globulin fraction in sea-bass support this assumption (Saad *et al*., 2013).

Our results revealed also non-significant changes in liver enzymes, urea, uric acid and creatinine in all experimental groups including the supplemented group with NS. Such findings confirm the safety of NS in aquaculture that is confirmed by other researchers (Saad *et al*., 2013 and Mohammed and Arias, 2016). The high content of essential oils in NS is recognized as safe admitted by the Food and Drug Administration (FDA, 2004).

The present study showed significant increase in antibody titer and phagocytic activity against *A. hydrophila* bacterin in catfish supplemented with NS. These findings were related to the recorded histopathological findings where excessive proliferation of the hematopoietic tissue together with marked activation of melanomacrophages cells in the parenchymatous organs were evident. A parallel study was in agreement with our study, where values of antibody titer, phagocytic index and activity increased significantly in sea bass vaccinated against *Pseudomonas florescence* bacterin supplementation of Black cumin seed and/or Turmeric (Saad *et al*., 2013). Additionally, the high relative level of protection (RLP) found among vaccinated and/or vaccinated-treated fish groups challenged with *A. hydrophila* indicate an immunostimulant effect of NS. Such findings are consistent with other research studies used Nigella sativa in other species as sea bass (Saad *et al*., 2013); Nile tilapia (Diab *et al*., 2008); rainbow trout (Awad *et al*., 2013).

In fish, antibody-mediated humoral immunity plays an important role in defending bacterial infections. Several mechanisms may share in the disease protectant effects noticed for NS in this study. These mechanisms include 1) the White blood cells of fish which play an important role in the cellular immunity and resistance to infectious diseases (Whyte, 2007) and Aly *et al*., 1998) globulin proteins and the phagocytic activities of fish phagocytes (Elkamel and Mosaad (2012) and Zhang, 2014) enhanced T cell immunity and production of cytokines (Haq *et al*., 1995), activation of lysozyme, total protein, antiprotease and bactericidal activity (Awad *et al*., 2013 and Swann L. and White, 1989) natural killer cell and compliment is also proposed (Mahdi, 1993). Since Immunostimulants, when used alone, amplify the specific immune response by elevating circulating antibody titers and numbers of plaque-forming cells (Anderson, 1992). and increase the immunocompetence and disease resistance of fish (Sakai, 1999), based on the current study, NS has immunomostimulating effect in vaccinated in catfish.

CONCLUSION

Supplementation of *Nigella sativa* at 3 g NS/100 g feed enhance the immune response and resistance of African catfish to *A. hydrophila* and therefore may have a potential value for pisciculture.

Ethical approval

All the animals were maintained in accordance with the National and International Institutional Guidelines for the Care and Use of Animals for Scientific purposes.

Competing interests

The authors declare that they have no significant competing financial, professional or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

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