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Effect of Black Cumin Seed Oil (*Nigella sativa*) on Enhancement of Immunity in the Climbing Perch, *Anabas testudineus*

Aisha Khatun[1], M. M. M. Hossain[1*], M. Z. Rahman[2], M. E. Alam[1], Farzana Yasmin[1], M.S. Islam[1] and M. M. Islam[1]

[1]Department of Fisheries and Marine Bioscience, Faculty of Biological Science and Technology, Jessore University of Science & Technology, Jessore-7408, Bangladesh.
[2]The Income and Nutrition (AIN) Project, The World Fish Centre, Jessore, Bangladesh.

Authors' contributions

This work was carried out in collaboration between all authors. Author MMMH designed the study, wrote the protocol and wrote the first draft of the manuscript. Author AK managed the literature searches, analyses of the study performed the spectroscopy analysis and author FY managed the experimental process author MZR helps to get fish samples from the different fish farmers and hatchery, author MEA managed laboratory works with different equipments adequately and authors MSI and MMI identified the species of plant. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: The study was conducted to examine the efficacy of dietery black cumin seed oil (*Nigella sativa*) on the immune response of climbing perch, *Anabas testudineus* against *A. hydrophila.*
Place and Duration: This experiment was performed in the Laboratory of Fisheries and Marine Bioscience (FMB), Jessore University of Science and Technology (JUST), on July to December 2013.
Methodology: Fish husbandry and experimental design, Culture and *Aeromonas hydrophila* Isolation, Diet Preparations, Serum preparation (immune response assay), Growth performance, bactericidal activity, phagocytic activity and challenge test have been performed in this study.

**Corresponding author: E-mail: mmiron_bau@yahoo.com;*

Results: Climbing perch (*Anabas testudineus*) of average weight 25±5 g were fed for 1, 2 and 4 weeks with diet supplemented 20 ml (20%), 30 ml (30%) and 40 ml (40%) 100 g^{-1} of *N. sativa* oil and with normal diet as control (0%). Immunological parameters including bactericidal activity and phagocytic activity were investigated. Treatment groups recorded enhancement in those parameters compared to the control. Treatment groups fed the dose 30% *N. sativa* oil showed a significant enhancement in bactericidal activity and phagocytic activity. The highest weight gain (WG) 41.7±1.5 was significantly increased with the 30% dose of *N. sativa* oil but specific growth rate (SGR) and feed conversion ratio (FCR) did not change significantly when compared to the control. Feeding with 30% dose diet to *A. testudineus* showed lowest cumulative mortality 20% compared to other dose diets and played most effective performance during challenge test.
Conclusion: This result suggests that 30% dose of *N. sativa* oil enriched diet significantly enhanced the immune response and disease resistance of *A. testudineus* against *A. hydrophila.*

Keywords: Anabas testudineus; Nigella sativa; Aeromonas hydrophila; disease resistance; bactericidal activity; phagocytic activity.

1. INTRODUCTION

Stress conditions are responsible for fish disease in aquaculture which leads to bacterial disease. Uses of antibacterial drugs in aquaculture are risky due to cross resistance against pathogens, toxicity, residues in tissues and contaminate the environment with bioactive product [1]. Using of natural plants as immunostimulant in fish is more useful than antibacterial drugs [2,3]. Medicinal plant as immunostimulants can be used not only against disease but also as growth promoters [4, 5]. From ancient time plants or plant products are used as medicine or therapeutic agent and it is well known [5]. *Nigella sativa Linn*. commonly known as the black cumin seed, is an annual herb that belongs to the botanical family of Ranuculaceae is a spice and preservative [6]. The seeds of *Nigella sativa* have been used for medicinal purposes as a natural remedy for a number of illnesses and conditions that include bronchial asthma, rheumatism, hypertension, diabetes, inflammation, cough, headache, eczema, fever and influenza [7]. Research has been conducted on immunomodulatory effect of *Nigella sativa* as an anti-tumor, bactericide, anticestode, antinematode, anti-inflammatory, analgesic, anti-diabetic and on some immuno-hematological parameters and specific as well as non-specific defence mechanisms of fish and also used as a growth promoter [3,8-13]. However, there was no report of this herb as immunostimulants in thai koi or *Anabas testudineas* to prevent diseases. Therefore, the aim of the present study was to examine if black cumin seeds, *Nigella sativa,* extract would influence some immunological parameters and immune response of *A. testudineas*.

2. MATERIALS AND METHODS

2.1 Fish, Experimental Design

Climbing perch or Thai koi of average weight 25 ± 5 g were collected from commercial fish farm and hatchery in Jessore and acclimatized in aerated freshwater (temperature maintained at 25 – 26°C) in the aquarium environment for 7 days before starting the experiment and during this time fish were fed twice daily with commercial diet. Length and weight were randomly checked initially upon receipt. Fish were randomly distributed into 4 groups each containing 10 fish representing three replicates and fed for 30 days with *N. sativa* oil enriched diet at 0% (control), 20%, 30% and 40% and oil was obtained by milling. To avoid dryness, evaporation of oil, lowering binder activity 20 to 40% were considered in substitution of 0.0 to 10% of *N. sativa* oil. Initially in this study the concentrations of *N. sativa* oil were tested 0 – 50% and bacterial colony against oil were found in the challenge experiment with 20 to 40% and therefore were chosen. The control group was fed with commercial diet to examine the possible mode of action and effect on immune response. Fish were fed at a rate of 3% to 2% body weight for twice in a day until the end of the experiment.

2.2 Culture and *Aeromonas hydrophila* Isolation

A. hydrophila isolated from diseased Climbing perch or Thai koi, was used in FMB laboratory for the study. Stocks were grown in brain heart infusion (BHI, Hi-media, Indian) and nutrient broth for 24 hrs at 37°C and then kept in −20°C until use. The subculture was taken and

centrifuged (4000 rpm for 15 min), after centrifugation the supernatant was discarded and the pellet was resuspended in sterile phosphate buffer saline (PBS). The culture was adjusted at 3.5×10^7 colony forming units (CFU) ml^{-1} by 10 times serial dilution and incubated at 37°C for 24 hours. The bacterium was confirmed by some biochemical test (Table 1).

2.3 Diet Preparations

Nigella sativa or black cumin seed was collected from local market, Borobazar in Jessore. The seed was dried at 40°C for 4 hours. Black cumin seed oil was extracted by milling the seed. Oil was flamed to make it disinfection and mixed with the commercial diet. The proximate composition of commercial diet and fatty acid analysis [16] of black cumin seed oil were shown in Table 2. Three experimental diets were prepared of the pellet with 20%, 30% and 40% of *N. sativa* oil were spread to the basal diet and mixed properly. To avoid dryness, evaporation of oil, lowering binder activity 20 to 40% were considered in substitution of 0.0 to 10% of *N. sativa* oil. Prepared feed with *N. sativa* was stored in a closed jar at room temperature.

2.4 Serum Preparation (Immune Response Assay)

Blood from the randomly selected fish were drawn directly from the caudal vein with the help of a sterilized 1 ml hypodermal syringe containing EDTA (Ethylene-Diamine-Tetra-Acetic Acid) as an anticoagulant using 24 gauge needles. For serum separation blood was collected without anticoagulant in serological tubes and stored in a refrigerator overnight. The clot was then spun down at 4500 g for 10 min. The collected serum was stored in sterile serum tubes at −20°C until used for assays. All the procedures were carried out in the sterilized condition. After drawing blood fishes were given 1% KMnO4 dip treatment and released in to the tank. For each group (0%, 0.5%, 1.0%, 1.5% and 2.0%) three culture plates were prepared. Bacterial stock solution was serial diluted for 10 times and 10^{-3}, 10^{-4} and 10^{-5} concentration were selected for further usage. Then 25 µl volume from each (10^{-3}, 10^{-4} and 10^{-5}) diluted solution was mixed with 25 µl separated serum (followed by disc diffusion method) of five different groups of fishes then spreaded in different culture plates and finally all plates were placed in an incubator at 37°C for 24 hrs. Then bacterial colonies of all plates were counted.

Table 1. Identifying characteristics of fish pathogenic bacteria *Aeromonas hydrophila*

Identifying characteristics	*Aeromonas hydrophila*
Colony	Yellowish
Morphology	Small rods
Gram strain	-
Catalase	+
Oxidase	+
Gelatin liquefaction	+
Indole production	+
OF test	F
Arabinose	+
Manitol	+
Sucrose	+
Inositol	+
Esculin hydrolysis	+
Voges-proskauer reaction	+
Ammonium production	-
Glucose	G

Note: + = positive reaction; - = negative reaction; O = oxidation; F = fermentation; G = gas

2.5 Growth Performance

All fish were deprived of food for 24 hour before weighing and sampling. Following parameters were measured for growth performance of Thai koi according to Choudhury et al. 2005 [17].

Weight gain (%) = Final body weight (g) – Initial body weight (g)/Initial body weight × 100
Specific growth rate (SGR) = Final weight (g) – Initial weight (g)/Time interval (days)
Feed conversion ratio (FCR) = Feed intake per body weight/Weight gain

2.6 Bactericidal Activity

A. hydrophila was used to examine the effectiveness of supplements to kill the bacterial infection. To prepare stock solution of experimental bacterial strain in conical flask containing 100 ml distill water, inoculating loop was touched from single bacterial colony of fresh culture. Bacterial suspension was then diluted using disk diffusion method. 15 µl of serum was added with 15 µl of bacterial suspension and mixed properly. The serum-bacterial mixture (15 µl) was plated onto the nutrient agar and BHI agar plates and incubated for 24 hours at 37°C before the numbers of colonies were counted.

2.7 Phagocytic Activity

Phosphate buffer solution **(**PBS) was fixed with gluteraldehyde and 6% suspension of thai koi blood cells were mixed in it. 20 µl of bacterial suspension was placed on a coverslip incubated for 30 min in a humid chamber. Then it was carefully washed with PBS and 20 µl of blood cells was added and incubated for 40 min. After staining with giamsa the numbers of engulfed blood cell or phagocytic cell were determined by photographic microscope (Axiocam ERc 5s with Axio vixim driver, Carl Zeiss, Germany).

2.8 Challenge Studies

For the challenge test virulent *A. hydrophila* strain were prepared from maintaining the serial dilution. On 30th day of feeding each group fishes were injected intraperitoneally (i.p.) with 0.5 ml of 24 hours cultured *A. hydrophila* which contained 3.2×10^6 CFU ml^{-1} challenge strain. The clinical signs and mortality was recorded up to 30 days of post challenge. The cumulative mortality was calculated by following Amandi, et al. 1982 [18] and Relative Percent Survival (RPS) was calculated as follow

$$\text{RPS} = 1 - \frac{(\%\ \text{Mortality in treated group})}{(\%\ \text{Mortality in control group})} \times 100$$

2.9 Statistical Analysis

Values for each parameter measured were expressed as the arithmetic mean ± standard error (SE). Effects of herbal diets on growth performance, hematological, and immunological parameters were tested using one-way ANOVA and the mean values were compared by using Duncan's multiple range tests at 5% level of significance [19].

3. RESULTS

3.1 Disease Resistance (Challenge)

The highest cumulative mortality was 80% in groups fed with 0% *N. sativa* enriched diets. The lowest mortality of 20% was noted in groups fed with 30% *N. sativa* enriched diets against *A. hydrophila* infection while 53% and 33% mortalities were observed when fed with 20% and 40% enriched diets. The survivality increased to 80% with 30% dose diet. Very low survivality 20%, 46% and 66% were found in 0%, 20% and 40% diets (Figs. 1 and 2).

3.2 Bactericidal Activity

The lowest number of bacterial colonies represented that the bacteria was resistant to the certain dose of *N. sativa* oil and efficiency of immune cells in serum to resist the bacteria. With *N. sativa* the lowest number of colonies (9×10^{-6}) was observed with the 30% dose compared to the control (85×10^6) (Fig. 3).

3.3 Phagocytic Activity

Phagocytic activity did not significantly enhance with 20%, 30% and 40% with enriched diet on first week against *A. hydrophila*. With 30% dose the activity significantly increased on 2 and 4 weeks, but not in 20% and 40% as compared with the control (Fig. 4).

3.4 Growth Performance

Fish in each aquarium were counted and group weighed every 2 weeks, following 24 h of feed deprivation. When fish were removed for weighing, aquaria were cleaned thoroughly, two-third of the water removed. Fish were not fed on sampling days. In Thai koi fed with all doses (20%, 30% and 40%) of supplementary diet growth rate significantly increased as compared to the control. The highest weight gain 41.5 ± 1.5 was found in 30% dose of *N. sativa* oil. However, the specific growth rate (SGR) and feed conversion ratio (FCR) did not significantly increased with any supplementation diet (Table 3).

4. DISCUSSION

Use of plant products in aquaculture industry has been reported to be safe as they are highly biodegradable and do not have any side effects such as drug resistance as observed with synthetic antibiotics [2]. *Nigella sativa* has immunomodulatory effect on fish against bacterial pathogen. Many researches has been conducted on immunomodulatory effect of *Nigella sativa* as an anti-tumor, bactericide, anticestode, antinematode, anti-inflammatory, analgesic, anti-diabetic and on some immuno-hematological parameters and specific as well as non-specific defense mechanisms of fish [3,8-13, 20]. In the present study, the obtained results indicate that final weight and weight gain (WG) are increased in treatment group compared to the control group. Highest weight gain is observed from the dose of 30% black cumin oil

with supplemented diet. The present results are similar to those reported by [8,21]. Significant increase in body weight and total biomass production as well as growth performance were seen in *Oreochromis niloticus* treated with 1.00 ppt stand for *Echinacea sp* [9]. Kelp grouper fed with all doses diet had significantly increased growth rate when compared to the control [22]. Moreover, Feeding rainbow trout with 1% lupin, *Lupinus perennis*, mango, *Mangifera indica*, or stinging nettle *Urtica dioica*, for 14 days led to significant enhancement in weight gain, SGR and FCR compared to the controls [23].

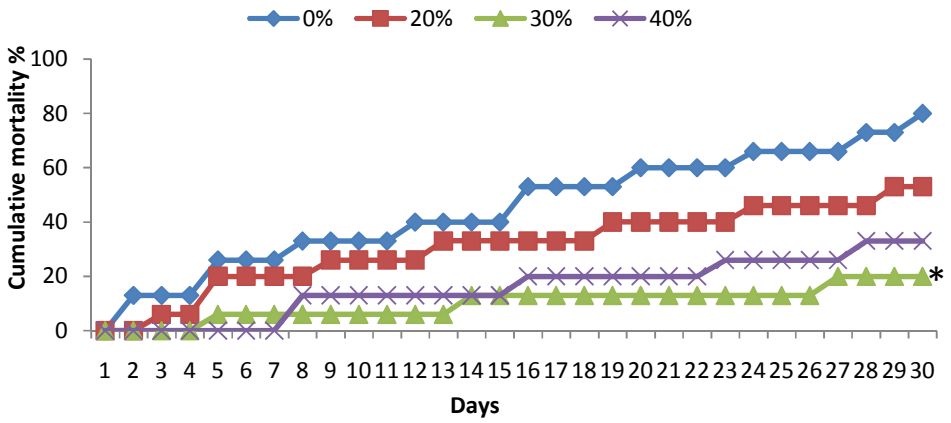

Fig. 1. Cumulative mortalities of *A. testudineus* fed supplementary diets with *N. sativa* oil and challenged with *A. hydrophila* (3.2×10^6 CFU ml^{-1}) for 30 days. (*) indicates relatively significance ($P < 0.05$)

Table 2. Proximate composition of supplementary feed, *N. sativa* and oil

Supplementary fish feed (%)		*N. sativa*		Fatty acid of *N. sativa* oil	
Protein	34	Protein	20.85	Linoleic acid	56
Crude fibre	6	Fat	38.20	Oleic acid	24.6
Crude ash	18	Moisture	4.64	Palmitic acid	12
Moisture	11	Ash	4.37	Stearic acid	3
Lipid	6			Eicosadienoic acid	2.5
Fat	3			Linolenic acid	0.7
				Myristic acid	0.16

(Source: Spectra fish feed Co. Ltd.), [14,15]

Table 3. Growth parameters of *A. testudineus* fed with different doses of *N. sativa* supplementation diet against *A. hydrophila*

Growth parameter	Doses	Week one	Week two	Week four
WG	0%	25.2±1.1	26.4±1.2	27.3±1.4
	20%	27.3±1.4	29.5±1.3	33.4±1.3
	30%	32.4±2.0	34.2±1.3	41.5±1.5 *
	40%	30.4±1.3	33.2±1.5	37.4±1.6
SGR	0%	1.2±0.2	1.3±0.1	1.4±0.2
	20%	1.3±0.2	1.4±0.1	1.5±0.2
	30%	1.5±0.3	1.6±0.4	1.7±0.3 *
	40%	1.4±0.2	1.5±0.3	1.6±0.1
FCR	0%	1.5±0.3	1.6±0.2	1.7±0.4
	20%	1.5±0.2	1.6±0.1	1.6±0.3
	30%	1.2±0.2	1.3±0.4	1.4±0.3 *
	40%	1.4±0.1	1.5±0.2	1.6±0.3

Data expressed as mean ± SE, (*) showed relatively significance ($P < 0.05$). Here, WG – Weight gain, SGR – Specific Growth Rate, FCR – Food Conversion Ratio

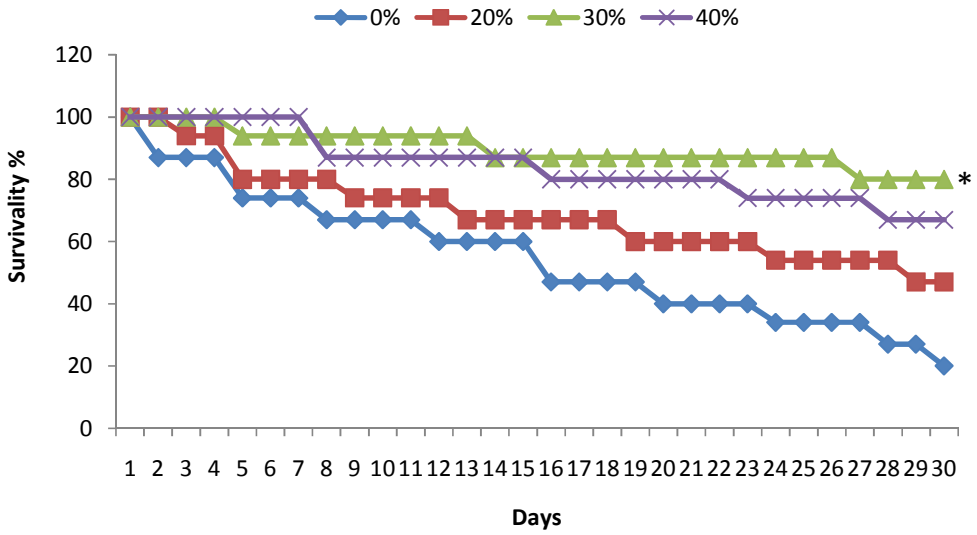

Fig. 2. Survivality of *A. testudineus* fed supplementary diets with *N. sativa* oil and challenged with *A. hydrophila* (3.2×10^6 CFU ml^{-1}) for 30 days. (*) showed relatively significance ($P < 0.05$)

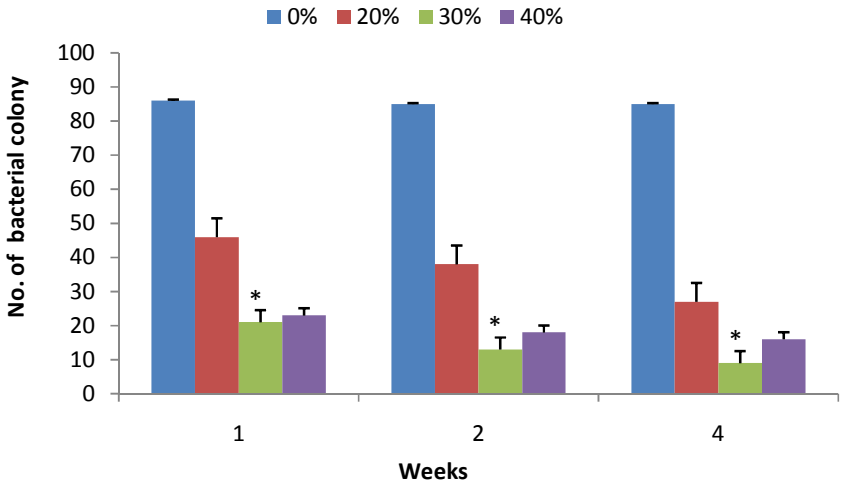

Fig. 3. Bactericidal activity (%) of Thai koi fed supplementary diet with 20%, 30% and 40% of *N. sativa* oil. (*) showed relatively significant ($P < 0.05$)

Serum bactericidal activity is a mechanism that helps to resist the growth of pathogen [24]. The lowest number of bacterial colonies indicated the efficiency of immune cells in serum to kill the pathogen. In the present study, the lowest numbers of bacterial colonies were observed in the treatment group than the control group especially at the dose of 30% of black seed oil. Awad et al. 2013 [13] who found greater serum bactericidal activity in rainbow trout (*Oncorhynchus mykiss*) administered with dietary supplements comprising 1% of Quercetin and 3% of *N. sativa* oil in his research study. Similarly, serum bactericidal activity has enhanced in catla (*Catla catla*) feeding the dietary supplements comprising prickly chaff-flower seed (*Achyranthes aspera*) [25]. Similar result was found in rohu (*Labeo rohita*) administered with prickly chaff-flower seed (*Achyranthes aspera*) [26].

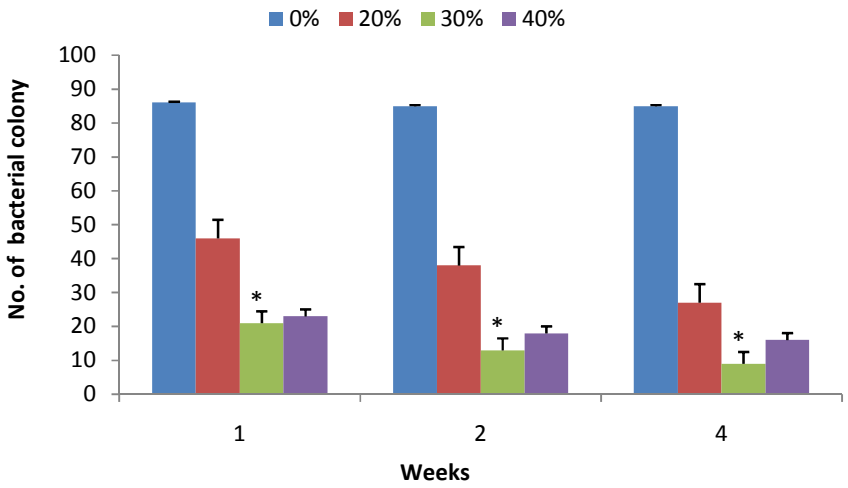

Fig. 4. Phagocytic activity (%) of *A. testudineus* fed with different doses of *N. sativa* extract against *A. hydrophila.* Data are significantly different (*P < 0.05*) from control at the same time by asterisks (*)

Phagocytic activity is increased by immunestimulant has been documented by many researcher [27-29]. In this study, the phagocytic activity was significantly increased in treatment group fed with 30% dose of *N. sativa* enriched diets from 1 to 4 week compared to the control group. Rainbow trout fed with 1% of stinging nettle and garlic recorded higher phagocytic activity than in the 0.1% dose, which was greater than the controls [28]. In olive flounder significantly increased phagocytic activity against *A. hydrophila* after being fed with 0.1% and 1.0% *Hericium erinaceum* enriched diets from 1 to 4 week [30]. Similar result was found in olive flounder fed with *Prunella vulgaris* [30]. Yin et al. 2006 [29] found that feeding tilapia with Scutellaria extract with higher doses (0.5 and 1.0%) caused reduction of function in phagocytic cells, while, when fish were fed with low dose of Scutellaria (0.1%) there was no stimulation on phagocytic activities.

In the present study after challenge with *A. hydrophila* (3.2×10^6 CFU ml^{-1}) mortalities were significantly reduced in all groups compared to the control group. The lowest mortality 20% was observed at the dose of 30% of *N. sativa* oil. But in 40%, mortality rate was increased because of suffocation due to overdose of oil. However, this result is in agreement with previous study conducted in *L. rohita* fed with *Achyranthes aspera* diet [31]. *O. mossambicus* treated with *Eclipta alba* leaf extract [32] against *Aeromonas hydrophila* infection.

Black cumin seed (*N. sativa*) is an immunomodulator and it exhibits an effective moderate activity in this result. It can be concluded that black cumin seed can be used as an antimicrobial drug for immunity enhancement and increasing survivality in farmed fish that are more susceptible to disease. However, because of its availability, low cost and immunostimulatory effect, it could be recommended to be used for fish to decrease mortalities caused by *Aeromonas hydrophila*.

5. CONCLUSION

The present study showed that 30% dose of *Nigella sativa* oil could significantly enhance immune response and reduce mortality and increase survival rate after challenge with the *Aeromonas hydrophila*. Thus, it can be deduced that using *N. sativa* oil as an immunestimulant in *Anabas testudineus* showed an immunity enhancement [33,34] which suggests a promising role for supplements as immunomodulatory components in fish feed help to make immune response in cultured fish against *A. hydrophila*. Further study needs to optimize the concentration of diet dose to prepare the herbal extract to control *A. hydrophila* infection in fish properly.

CONSENT

It is not applicable.

ETHICAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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