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SHORT COMMUNICATION KISA BİLGİLENDİRME

THE EFFECTS of *Nigella sativa* OIL ON THE IMMUNE SYSTEM OF RAINBOW TROUT WITH DIFFERENT APPLICATION METHODS

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Abstract: In this study, changes in some non-specific immune defense mechanisms of rainbow trout (*Oncorhynchus mykiss*) following exposure to aqueous extracts of *Nigella sativa* seeds were investigated. A total of 900 fish (mean weight 102.3g) were used in the study. Fish were treated with black seed oil using one of three different application techniques. The first method involved feeding the fish with 0.1%, 1.0% and 10% dilution of black seed oil over a 21d period. In the second method, 0.1%, 1.0% and 10% dilution of black seed oil was injected to the fish. In the third method, 0.1%, 1.0% and 10% dilution of black seed oil were applied directly onto the lateral line of fish ('smearing') over a 3d period. Blood samples of fish were taken at 3, 7, 14 and 21d post-exposure and hematocrit, leucocrit, erythrocyte, leucocyte, nitroblue tetrazolium activity, protein level and total immunoglobulin levels were determined. The highest levels of hematocrit, erythrocyte, leucocyte and nitroblue tetrazolium activity occurred in fish exposed using the smearing method. Differences in leucocrit levels of groups exposed to the three methods were not significant. The highest levels of total protein occurred in fish exposed via feeding. The highest levels of total immunoglobulin occurred in fish exposed via injection and decreased suddenly. Nonetheless, the smearing method caused regular increase and decrease. It was observed that black seed oil caused increase of fish protein content, even at the lowest dose in fish exposed via feeding. In this study, the effects of smearing method were investigated on immune system of rainbow trout, as well as, determined differences of feeding and injection methods on immune response than smearing method.

Keywords: Black seed oil, Rainbow trout, *Nigella sativa*, *Oncorhynchus mykiss*, Non-specific immune parameters, Immune system of fish.

FÜBAP 1501 nolu proje desteği ile yapılan doktora tezinden özetlenmiştir.

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Özet:

Çörekotu (*Nigella sativa*, L) Yağı' nın Gökkuşağı Alabalığı (*Oncorhynchus mykiss*, Walbaum, 1792)'nın İmmün Sistemine Etkisinin Araştırılması

Bu çalışmada çörekotu (*Nigella sativa*, L) yağı'nın gökkuşağı alabalığı (*Oncorhynchus mykiss*, Walbaum, 1792)' nın spesifik olmayan immün sistem parametrelerine etkileri araştırıldı. Araştırmada ortalama ağırlığı 102.30 g (84.2–121.1 g) olan 900 adet gökkuşağı alabalığı (*Oncorhynchus mykiss*) kullanıldı. Çalışmada çörekotu yağı alabalıklara 3 farklı yöntemle verildi. 1. yöntemde çörekotu yağı %0.1, %1.0 ve %10 oranlarında yeme ml. olarak ilave edilerek 21 gün süreyle alabalıklara verildi. 2. yöntemde çörekotu yağı %0.1, %1.0 ve %10 oranlarında balık dozları enjeksiyonla alabalıklara uygulandı. 3. yöntemde çörekotu yağının % %0.1, %1.0 ve %10 dozları 3 gün boyunca alabalıkların yanal çizgisine sürülerek uygulandı. Bu işlemler sonrasında alabalıklardan, 3, 7, 14, 21. günlerde kan örnekleri alındı. Alınan bu örneklerin hematokrit (Ht), lökokrit (Lt), eritrosit, lökosit, nitroblue tetrazolium (NBT) aktivitesi, Protein düzeyi ve toplam immünoglobülin seviyeleri belirlendi. Sonuç olarak, hematokrit, eritrosit, lökosit, NBT seviyelerinin en yüksek seviyeleri, çörekotu yağının balıklara sürülerek uygulamasında tespit edildi. Üç yöntemin uygulandığı farklı grupların lökokrit seviyelerinde kayda değer bir değişim bulunmadı. Toplam protein seviyeleri ölçümlerinde besleme yöntemi uygulanan gruplarda en yüksek değerler elde edildi. Toplam Ig seviyeleri incelemelerinde en yüksek değerler enjeksiyon yönteminde elde edildi fakat parabolik bir çizgi izlemediği bununla birlikte sürme uygulamasında parabolik bir eğri tespit edildi. Besleme yöntemi ile verilen gruplarda çörekotu yağının besleyici özelliklerinin minimum oranlarda bile kullanıldığında protein seviyelerini yükseltecek kadar etkili olduğu tespit edildi. Bu çalışmada sürme yönteminin bağışıklık sistemine etkisinin yanı sıra enjeksiyon ve besleme yönteminin immün cevapları arasında farklılığı tespit edilmiştir.

Anahtar Kelimeler: Çörek otu yağı, Gökkuşağı alabalığı, *Nigella sativa*, *Oncorhynchus mykiss*, Spesifik olamayan bağışıklık parametreleri, Balık bağışıklık sistemi

Introduction

The use of immunostimulants as an alternative to the drugs, chemicals and antibiotics currently being used to control fish diseases in fish culture has a limited success rate. After all, herbs have perfectly complex matters (tanens, terpenes, aldehides, aromatic hydrocarbons, etc.). The complex has both immunostimulant and complemental materials necessary for body (Logambal *et al*., 2000; Yunxia *et al*., 2001).

At present, the treatment of fish diseases and prophylaxis uses both traditional methods and dietary supplementation, injection and bathing. The use of feeding methods to treat various fish ailments with antibiotics and chemical immunostimulants is insufficient. The most common method of all is the primary injection and secondary bath method. However these methods are costly in terms of manpower and may also cause increased stress to fish in comparison to basic feeding methods (Alberto *et al.*, 2005).

Immunostimulants in herbs contain compounds that are passed directly through fish skin. Having immunomodulatory materials in Black seed (*Nigella sativa*) is considered to be one of the greatest healing herbs. Black seed oil is thought to have both immunomodulatory and immunostimulant properties. Synthetic stimulants used in aquaculture are deposited in fish tissue and are potentially passed indirectly to human consumers. For this reason, organic, herbal products may be prefered as fish immunostimulants (Al–Ghamdi, 2001; Badary et al., 1997; Badary and Gamal, 2001; Islam *et al*., 2004).

Materials and Methods

A total of 900 rainbow trout (*Oncorhynchus mykiss*) of 102,30g mean initial body weight were obtained from the Çırçır Rainbow Trout Fish Farm (Keban/Elazığ, Turkey). Fish were maintained in re-circulating tanks for adaptation for one week. The fish were then divided into four groups (one control and three experimental groups), each comprising 50 fishes, and exposed to one of three methods (diet, intra-peritoneal injection and smeared up at lineal lateral). Experimental groups were fed diet at a rate of 2% fish body weight per day for a period of three weeks.

At the end of study period, fish were anaesthetized with Benzocaine (50 ppm) and blood was taken by caudal vein puncture with heparinized syringes. Hematocrit (Ht), Leucocrit (Lt), Erythrocyte, Leucocyte, Nitroblue Tetrazolium (NBT) activity, Protein level and Total immunoglobulin levels were determined. All data were analyzed using SPSS and Microsoft Excel. Experimental groups are detailed in Table 1.

Table 1. Experimental groups

Methods Feeding	**Experimental groups**
	1- Control of diet feeding method (No added Black seed oil)
	2- 0.1% Black seed oil added diet
	3- 1.0% Black seed oil added diet
	4- 10.0% Black seed oil added diet
I.p. injection	1- Placebo control of i. p. injection group (Nothing added)
	2- Placebo control of black seed oil group (Injected with sunflower oil only)
	3-0.1% dose of black seed oil was injected to the fish (Diluted with Sunflower oil)
	4- 1.0% dose of black seed oil was injected to the fish (Diluted with Sunflower oil)
	5- 10.0% dose of black seed oil was injected to the fish (Diluted with Sunflower oil)
Smeared up lateral line	1. Control group: only sunflower oil were smeared up lateral line of fish (No Black seed oil used)
	2. Control group: black seed oil was smeared up lateral line of fish (Only sunflower oil)
	3. Group: 0.1% black seed oil was smeared up lateral line of fish. (Diluted with Sunflower oil)
	4. Group: 1.0% black seed oil was smeared up lateral line of fish (Diluted with Sunflower oil)
	5. Group: 10.0% black seed oil was smeared up lateral line of fish (Diluted with Sunflower oil)
Days	a: 3. day, b: 7. day, c: 14. day, d: 21. day

Results and Discussion

Leukocyte levels in the fish fed with 0.1%, 1.0% and 10.0% black seed oil added diet were found to be different compared to control group ($P<0.05$). Leukocyte levels in the fish with 10.0% black seed oil i. p. injection was found to be different compared to control group ($P<0.05$) but another were not different. Leukocyte levels in the fish smeared up lateral line with 0.1%, 1.0% and 10.0% black seed oil were found to be different compared to control group and leucocyte levels of other groups (Table 2).

Erythrocyte levels in the fish fed with 0.1%, 1.0% and 10.0% black seed oil added diet were not found to be different compared to control group ($P>0.05$). Erythrocyte levels in the fish with 10.0% black seed oil i. p. injection were found to be different when compared with the control group at the 14. day. Erythrocyte levels in the fish smeared up lateral line with 0.1%, 1.0% and 10.0 % black seed oil were found to be different when compared with control group and leucocyte levels of other groups (Table 3).

Haematocrit and leucocrit levels in the fish fed, i. p. injection and smeared up lineal lateral

with black seed oil weren't found to be different compared to control group.

NBT levels in fish fed with 0.1%, 1.0% and 10.0% black seed oil added diet were not found to be different when compared to the control group. But at the 14. day, these levels were increased. NBT levels in fish with 0.1% and 10.0% black seed oil i. p. injection were found to be different compared to control group at the 14. Day ($P>0.05$) and later were decreased. The most NBT levels in the fish smeared up lateral line with 10.0% black seed oil were found to be different when compared with the control group and NBT levels of other groups as long as 21 days (Table 4).

Total protein levels in fish fed with 0.1%, 1.0% and 10.0% black seed oil were found to be different when compared with the control group and total protein levels of other groups as long as 21 days (Table 5).

Total Ig levels in the fish i. p. injection with black seed oil was found to unstable increased compare to control group and Total Ig levels of other groups as long as 21 days. But, Total Ig levels of fish in the black seed oil smeared up lateral line of fish was showed a parabolic line. (Table 6, 7).

Table 2. Leucocyte levels in Rainbow trout black seed oil were smeared up lateral line of fish (Diluted with Sunflower oil).

Groups	a	b	c	d
1	27.62 ± 0.58	27.95 ± 0.95	30.24 ± 1.01	26.77 ± 0.94
2	25.14 ± 0.42^{1a}	26.76 ± 0.78^{1,3,4a,1b}	25.56 ± 0.49^{1,3,4a,1,5b,1c}	25.14 ± 0.46^{1,3,4a,1,5b,1,3,5c}
3	30.06 ± 1.41^{1,2a}	25.16 ± 0.33^{1,3,4a,1b}	28.38 ± 0.61^{2,3,5a2,3,4b,2c}	21.53 ± .62^{1,3,4,5a,1,2,3,4,5b,1,2,3,5c,1,2d}
4	30.26 ± 1.23^{1a}	23.62 ± 0.61^{1,3,4a,1b}	23.57 ± 0.63^{1,3,4a,1,5b,1,3c}	24.28 ± 0.56^{1,2,3,4a,1,5b,1,3,5c,1,3d}
5	24.19 ± 0.73^{1,3,4a}	29.02 ± 0.64^{2,5a,2,3,4b}	28.49 ± 0.62^{2,3,5a,2,3,4b,2,4c}	26.05 ± 0.22^{3,4a,4,5b,3,4,5c,3d}

1: Control group: only sunflower oil were smeared up lateral line of fish (No Black seed oil used), 2: Control group: black seed oil were smeared up lateral line of fish. (only sunflower oil), 3: 0.1% black seed oil were smeared up lateral line of fish. (diluted with Sunflower oil), 4: 1.0% black seed oil were smeared up lateral line of fish (diluted with Sunflower oil), 5: 10.0% black seed oil were smeared up lateral line of fish (diluted with Sunflower oil). a: 3. day, b: 7. day, c: 14. day, d: 21. day.

Table 3. Erythrocyte levels in Rainbow trout treated with black seed oil smeared up lateral line of fish (Diluted with Sunflower oil).

Groups	a	b	c	d
1	25.0 ± 1.62	24.1 ± 1.73	25.7 ± 1.20	24.8 ± 1.02
2	50.2 ± 5.96	51.2 ± 3.76^{3,4a,1b}	50.8 ± 3.46^{3,4a,1,3,4,5b,1c}	53.2 ± 4.36^{3,4a,1,3,4,5b,1,3,4,5c,1d}
3	34.7 ± 3.82^{1a}	66.1 ± 4.18^{1,3,4,5a,1,2b}	81.1 ± 6.13^{1,3,4,5a,1,2,3,4,5b,1,2c}	92.9 ± 2.46^{1,3,4,5a,1,2,3,4,5b,1,2,3,4,5c,1,2d}
4	22.8 ± 1.42^{1,3a}	37.9 ± 2.19^{1,4a,1,2,3b}	70.2 ± 3.26^{1,2,4,5a,1,2,4b,1,2c}	72.0 ± 4.20^{1,3,4,5a,1,2,4b,1,2c,1,2,3d}
5	40.2 ± 2.61^{4a}	61.8 ± 4.73^{1,3,4,5a,1,2,4b}	70.1 ± 3.79^{1,3,4,5a,1,2,4b,1,2c}	102.0 ±5.16^{1,3,4,5a,1,2,3,4,5b,1,2,3,4,5c,1,2,3d}

1: Control group: only sunflower oil were smeared up lateral line of fish (No Black seed oil used), 2: Control group: black seed oil were smeared up lateral line of fish. (only sunflower oil), 3: 0.1% black seed oil were smeared up lateral line of fish. (diluted with Sunflower oil), 4: 1.0% black seed oil were smeared up lateral line of fish (diluted with Sunflower oil), 5: 10.0% black seed oil were smeared up lateral line of fish (diluted with Sunflower oil). a: 3. day, b: 7. day, c: 14. day, d: 21. day.

Table 4. NBT levels in Rainbow trout treated with black seed oil smeared up lateral line of fish (Diluted with Sunflower oil).

Groups	a	b	c	d
1	11.84 ± 0.62	12.18 ± 1.09	14.47 ± 1.22	10.99 ± 1.11
2	18.90 ± 0.88[1a]	14.46 ± 0.40[1,2,3,4a]	15.59 ± 1.06[1,2,3,4a,1,3,5b]	16.78 ±1.98[1,2,3,4a,1,3,5b,1d]
3	18.95 ± 1.12[1a]	11.47 ± 0.88[2,3,4a,2b]	14.35 ± 1.26[2,3,4a,3,5b]	22.85 ±0.75[1,2,3,4,5a,1,2,3,4,5b,1,2,3,4,5c,1,2d]
4	19.17 ± 0.62[1a]	16.07 ± 0.67[1,2,3,4,5a,1,3b]	15.69 ± 0.96[1,2,3,4a,1,3,5b]	18.87 ±1.13[1,5a,1,2,3,4,5b,1,2,3,4,5c,1,2,3d]
5	13.17 ± 0.30[2,3,4a]	13.74 ± 0.21[1,2,3,4,5a,1,2,3,4b]	13.94 ± 1.23[2,3,4a,5b]	15.26 ± 0.86[1,2,3,4a,1,3,5b,1,3,4d]

1: Control group: only sunflower oil were smeared up lateral line of fish (No Black seed oil used), 2: Control group: black seed oil were smeared up lateral line of fish. (only sunflower oil), 3: 0.1% black seed oil were smeared up lateral line of fish. (diluted with Sunflower oil), 4: 1.0% black seed oil were smeared up lateral line of fish (diluted with Sunflower oil), 5: 10.0% black seed oil were smeared up lateral line of fish (diluted with Sunflower oil). a: 3. day, b: 7. day, c: 14. day, d: 21. day.

Table 5. Total protein levels in Rainbow trout treated with black seed oil smeared up lateral line of fish. (Diluted with Sunflower oil).

Groups	a	b	c	d
1	0.53 ± 0.039	0.51 ± 0.087	0.52 ± 0.076	0.51 ± 0.069
2	0.50 ± 0.020	0.52 ± 0.033	0.51 ± 0.032[4a,4b]	0.50 ± 0.035[4a,4b,4c]
3	0.44 ± 0.027	0.57 ± 0.038[5a]	0.54 ± 0.020[3,4,5a,4,5b]	0.51 ± 0.028[4a,4b,4c]
4	0.63 ± 0.023[1,2,3a]	0.40 ± 0.028[1,2,5a,1,3b]	0.28 ± 0.020[1,2,3,4,5a,1,3,4,5b,1,2,3c]	0.64 ±0.027[1,2,3,5a,1,3,4,5b,1,2,3,4c,1,2,3d]
5	0.43 ± 0.028[1,4a]	0.45 ± 0.023[1,5a,1b]	0.56 ± 0.021[3,4a,4,5b,4c]	0.73±0.052[1,2,3,4,5a,1,3,4,5b,1,2,3,4,5c,1,2,3,4d]

1: Control group: only sunflower oil were smeared up lateral line of fish (No Black seed oil used), 2: Control group: black seed oil were smeared up lateral line of fish. (only sunflower oil), 3: 0.1% black seed oil were smeared up lateral line of fish. (diluted with Sunflower oil), 4: 1.0% black seed oil were smeared up lateral line of fish (diluted with Sunflower oil), 5: 10.0% black seed oil were smeared up lateral line of fish (diluted with Sunflower oil). a: 3. day, b: 7. day, c: 14. day, d: 21. day.

Table 6. Total Ig levels in rainbow trout black treated with black seed oil injected into the peritoneal cavity of the fish (i. p.). (Diluted with Sunflower oil).

Groups	a	b	c	d
1	11.84 ± 0.62	12.18 ± 1.09	14.47 ± 1.22	10.99 ± 1.11
2	13.58 ± 0.54	14.08 ± 0.74[3a]	14.57 ± 0.76[3,4a,3,5b]	13.04 ± 0.98[3,4a,3,5b,3,5c,1d]
3	21.04 ± 1.51[1,2a]	15.83 ± 0.46[1,2,3,4a,1,2b]	16.57 ± 0.77[1,2,3,4,5a,1,2,4,5b,2c]	16.13±0.65[1,2,3,4a,1,2,4,5b,2,5c,1,2d]
4	20.75 ± 1.12[1,2a]	13.17 ± 0.30[3,4a,3b]	16.64 ± 0.40[1,2,3,4,5a,1,2,4,5b,1,2c]	13.76 ±0.59[3,4a,5b,3,4,5c,1,3d]
5	14.31 ± 0.58[1,3,4a]	19.41 ± 0.28[1,2,5a,1,2,4b]	18.63 ± 0.58[2,3,5a,1,2,3,4b,1,2c]	15.23 ±0.3[1,3,4a,1,5b,5c,1d]

1: Plasebo control of black seed oil was injected to the fish (only injection, does not have oil and anything), 2: Plasebo control of black seed oil was injected to the fish (Sunflower oil), 3: % 0.1 dose of black seed oil was injected to the fish (Diluted with Sunflower oil), 4: % 1 dose of black seed oil was injected to the fish (Diluted with Sunflower oil), 5: % 10 dose of black seed oil was injected to the fish (Diluted with Sunflower oil). a: 3. day, b: 7. day, c: 14. day, d: 21. day.

Table 7. Total Ig levels in Rainbow trout treated with black seed oil smeared up lateral line of fish (Diluted with Sunflower oil).

Groups	a	b	c	d
1	271.4 ± 11.49	269.3 ± 14.23	270.4 ± 12.54	272.3 ± 12.48
2	202.8 ± 18.94[1a]	200.93 ± 13.04[4,5a,1b]	201.2 ± 13.94[4,5a,1,4,5b,1c]	200.82 ± 15.44[4,5a,1,4,5b,1,3,4,5c,1d]
3	206.9 ± 8.68	163.9 ± 9.78[4,5a,1b]	357.8 ± 21.64[1,2,3,4,5a,1,2,3b,1,2c]	486.9 ± 17.95[1,2,3,4,5a,1,2,3,4,5b,1,2,3,4c,1,2d]
4	137.8 ± 5.57[1,2,3a]	372.0±15.28[1,2,3,4a,1,2,3b]	355.0±17.33[1,2,3,4,5a,1,2,3b,1,2,4c]	472.1 ± 35.69[1,2,3,4,5a,1,2,3,4,5b,1,2,3,4c,1,2d]
5	353.8±8.10[1,2,3,4a]	315.1±23.60[1,2,3,4a,2,3b]	466.9±31.30[1,2,3,4,5a,1,2,3,4,5b,1,2,3,4c]	704.1±2.10[1,2,3,4,5a,1,2,3,4,5b,1,2,3,4,5c,1,2,3,4d]

1: Control group: only sunflower oil were smeared up lateral line of fish (No Black seed oil used), 2: Control group: black seed oil were smeared up lateral line of fish. (only sunflower oil), 3: 0.1% black seed oil were smeared up lateral line of fish. (diluted with Sunflower oil), 4: 1.0% black seed oil were smeared up lateral line of fish (diluted with Sunflower oil), 5: 10.0% black seed oil were smeared up lateral line of fish (diluted with Sunflower oil). a: 3. day, b: 7. day, c: 14. day, d: 21. day.

In this study, three different immunostimulant practice methods are used. Smeared up to linea lateral of fish that showed stimulated specific defense mechanisms of rainbow trout. Smearing black seed oil up the lateral line of individual fish proved to be the most efficacious method of all of those tested (also including dietary supplementation and i. p. injection). The highest levels of Erythrocyte, Haematocrit, Leucocyte, NBT were all recorded in fish exposed to the smeared method.

Having powerful structure of immune system cells are not permitted to diseased by means of pathogen microorganisms. Diseases are blocked with higher NBT and Total Ig levels than normal levels in fish (Blazer and Wolke, 1984).

Intra-peritoneal injection is the most commonly used method for boosting total Ig levels in the administration of commercial fish vaccines because of its reliability and high efficiency, and the results of our study support this (Sisson *et al*., 1997). But this method did not show parabolic equation. However, Total Ig levels of smeared method did show a parabolic equation, which is the desired situation for immunostimulatory methods because it indicates that immunostimulants accumulate over a long time in the tissue of fish.

Especially, the highest NBT and erythrocyte levels were recorded in smeared method that is showed transdermal affects some of immunostimulants as nigellin, nigellon and thymoquinone. The organs of hemapoietic and immune system are stimulated by these immunostimulants with transdermal pathway.

Serum protein level is an important indicator of humoral defense system of fish and increase especially in the fish fed with black seed oil.

Conclusion

It could be concluded that black seed oil could be recommended as an immunostimulant substance. It is also recommend using black seed oil by smearing up lineal lateral of fish for prophylaxis rather than for treatment.

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