Journal of Limnology and Freshwater Fisheries Research
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Immunostimulant Effects of Geophyte Plant Extract on Non-specific Defence Mechanisms of Rainbow Trout (*Oncorhynchus mykiss*)

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ABSTRACT

The aim of this study was to determine the immunostimulant effects of geophyte plant extract on non-specific defence mechanisms of rainbow trout (*Oncorhynchus mykiss*). For this purpose, *Muscari comosum* was collected in Muğla region and extracted in ethanol. Then, The plant extract applied into fish by intraperitonal injection in two different concentrations (0.5mg/ fish and 2.0 mg/fish). The average fish weight was 140 g. Following the injection on the 1st, 7th, 14th, 21st, 28th days the blood and serum samples were collected from fish in each group and examined for various parameters including percentage of hematocrit, the counts of nitroblue tetrazolium (NBT) positive neutrophils, total leukocyte counts, percentage of white blood cells and serum lysozyme activity. The results indicated that the counts of NBT (+) neutrophils, percentage of monocyte and neutophil and total leukocyte counts increased in the group with injected 0.5mg plant extract /fish compared to control group ($P<0.05$). It has been revealed that this dose of *M. comosum* can be suggested to use to enhance non-specific immune system for rainbow trout aquaculture. However, immunostimulant effects of oral administration of this plant extract in rainbow trout are needed to be determine in future studies.

Keywords: Fish, immune system, *Muscari comosum*, Tassel hyacinth, blood

ARTICLE INFO

RESEARCH ARTICLE

Received : 09.01.2018
Revised : 07.03.2018
Accepted : 09.03.2018
Published : 27.04.2018

DOI:10.17216/LimnoFish.376404

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Gökkuşağı Alabalığı (*Oncorhynchus mykiss*)'nın Spesifik Olmayan Savunma Mekanizması Üzerine Geofit Bitki Ekstraktının İmmunostimulant Etkisi

Öz: Bu çalışmada amacımız, gökkuşağı alabalığının spesifik olmayan savunma mekanizması üzerine geofit bitki ektraktının immunostimulant etkisinin belirlenmesidir. Bu amaçla geofit bir bitki olan *M. comosum* Muğla bölgesinden toplandı ve etanolde ekstrakte edildi. Daha sonra farklı dozlarında (0,5 mg/balık ve 2,0 mg/balık) balıklara intraperitonal olarak uygulandı. Balıklar ortalama 140 g ağırlığa sahipti. Deneme süresince uygulamayı takiben 1, 7, 14, 21, 28. günlerde herbir gruptaki balıktan kan ve serum örnekleri alınarak farklı parametreler (hematokrit yüzdesi, nitroblue tetrazolium (NBT) pozitif nötrofil sayısı, toplam lökosit sayısı, beyaz kan hücrelerinin yüzdesi ve serum lizozim aktivitesi) açısından incelendi. Bu çalışmanın sonucunda NBT pozitif nötrofillerin sayısı, monosit ve nötrofil yüzdeleri ve toplam lökosit sayısı 0,5 mg/balık dozunda injeksiyon yapılan grupta kontrol grubuna kıyasla artış gösterdiği ($P<0,05$) belirlendi. Bu nedenle gökkuşağı alabalığı yetiştiriciliğinde spesifik olmayan immun sistemi uyarmak için bu dozdaki *M. comosum*'un kullanılması önerilebilir. Ancak, bitki ekstraktının oral yolla yeme ilave edilerek balıklara uygulanması ile immunostimulant etkisinin belirlenmesi için gelecekte yeni çalışmalara ihtiyaç duyulmaktadır.

Anahtar kelimeler: Balık, immun sistem, *Muscari comosum*, arap sümbülü, kan

How To Cite
Uluköy G, Kubilay A, Didinen BI, Metin S, Altun S, Diler Ö, Mammadov R, Dulluç A, 2018. Immunostimulant Effects of Geophyte Plant Extract on Non-specific Defence Mechanisms of Rainbow Trout (Oncorhynchus mykiss). LimnoFish. 4(1): 36-41. doi: 10.17216/LimnoFish.376404

Introduction

Fish production has increased significantly over the past decades which has given rise to practices such as over crowding, transport, handling and grading in aquacultural systems. These practices are the major factors that make the fish susceptible

against pathogens. Diseases are major constraints to aquaculture production (Plumb and Hanson 2011)

Fish disease is rarely a simple association between pathogen, a host fish and environmental problems, such as poor water quality or other stressors often contribute to the outbreak of disease. Fish disease, caused by pathogenic organisms present in the environment, they are mostly contagious and treatment may be necessary to control the disease outbreak. Therefore, intensive farming practices and infectious diseases induced major problems in aquaculture industry causing heavy loss to farmers. Several studies have been conducted on the modulation of fish immune system in order to prevent the outbreaks. Disease outbreaks are increasingly being recognized as a potential constraint on aquaculture production and trade and cause massive financial loss either through mortality or reduced meat quality, resulting in reduced profit margins (Plumb and Hanson 2011; Mehana et al. 2015).

The use of antibiotics and chemotherapeutics to combat fish diseases has the risk of generating resistant pathogens, bioaccumulation and environmental pollution. Commercial vaccines are expensive for fish farmers and are specific against particular pathogens (Christybapita et al. 2007). One of the most promising methods of controlling diseases in aquaculture is strengthening the defence mechanisms of fish through prophylactic administration of immunostimulants (Deivasigamani and Subramanian 2016; Christybapita et al. 2007). The different types of IS (e.g. glucan, chitin, lactoferrin, levamisole, vitamin B, C, E and growth hormone and prolactin) mainly facilitate the function of phagocytic cells. Several immunostimulants also stimulate the natural killer cells (NK), complement, lysozyme and antibody responses of fish (Dügenci et al. 2003; Mehana et al. 2015). Up to now, several plants which have been used as therapeutic in the control of pathogens which cause diseases in fish. (Awad and Awaad 2017). Geophyte plant, *M. comosum* (L.) Mill.1768, has medicinal properties (Villa et al. 2012; Nasrabadi et al. 2013) such as diuretic, anti-inflammatory, hypoglycemic activities (Loizzo et al. 2010) and antioxidant activity (Pieroni et al. 2002). Further, *M. comosum* had been determined immunustimulant effects on gilthead seabream (*Sparus aurata*) by Baba et al. (2014).

The aim of the present study was to determine the immunostimulant effects of *M. comosum* extract on the nonspecific immune responses of rainbow trout.

Materials and Methods

Plant extraction

The geophyte plant, Tassel hyacinth (*Muscari comosum*), was gathered from Muğla region of Turkey. Plant brought to laboratory for the extraction. Its bulbs were cleaned and chopped into small pieces (Lee et al. 2000; Tanker and Tanker 1991). The extraction process was continue in ethyl alcohol at 50°C in a water bath for 24 h. The obtained solution was filtered and then remained mass was run again with applying same process as before. This step was repeated three times. The obtained extracts combined and lyophilized. The extract was stored in dark at 4°C.

Experimental design

Healthy rainbow trout (mean weight of 140±10g) were taken from a private fish farm in Isparta and acclimated for 2 weeks and held in 600 liters tanks filled with freshwater at 12°C, with a flow rate of 1-1.5 L min-1 with continuous aeration. Fish were fed with commercial pellets at 2% body weight and water quality parameters were monitored daily. The experimental fish were randomly divided into 3 groups, 50 fish/group in duplicate. The obtained lyophilized *M.comosum* extract was dissolved it in sterile phosphate-buffered saline (PBS) solution and then applied to fish with an intraperitoneal injection (i.p.) as 0.1 mL of two different concentrations as 0.5 mg/fish and 2 mg/fish. The same volume of PBS i.p. injected into fish of control group.

Sampling

The each sampling day (1st, 7th, 14th, 21st, and 28th days), randomly selected total five fish from each group were taken and anesthetized by using phenoxyethanol. After anasthesizing fish, blood samples were drawn from the caudal vein and then fish sacrificed. A part of blood was put into an Eppendorf tube and then left at 4°C overnight and centrifuged at 3500 g for 15 min and then serum collected. The serum samples were stored at −20°C until assayed.

Determination of NBT-positive cells

For the determination of the respiratory burst activity in the blood, Nitroblue tetrazolium (*NBT*) (Sigma No: N-6876) was used by following a modified method described by Anderson et al. (1992). A drop of blood placed onto a microscope coverslip and left in a petri dish which had humid atmosphere for 30 min at 25°C. After incubation, the coverslip washed with 0.067 mM sodium phosphate buffer (pH 6.4) to remove unwanted cells and then a drop of 0.2% NBT solution

(freshly prepared) was put on to slide and the coverslip with cells was turned cell face down on the NBT solution and incubated again in a petri dish for 30 min at 25ºC. The slides were examined under microscope (40x magnification). The dark blue staining cells were counted as positive. From each fish, five coverslips were prepared and five random microscopic fields were counted on each slide. Then the total twenty-five different fields were averaged and the mean and standard error of values per field for fish were calculated.

Assay of lysozyme activity

2 mg lyophilized *Micrococcus lysodeikticus* cells (Sigma, M 3770, ATCC No. 4698) was suspended with 10 ml 0.05 M sodium phosphate buffer (pH 6.5). The 3 ml of from the solution was taken the spectrophotometer cells and than added onto 50µl fish serum. Following this process after 30 seconds and 4.5 minutes was performed two measurements with Shimadzu (UV-120-02) spectrophotometer at 540 nm. One unit of lysozyme activity was defined as a reduction of absorbance of 0.001 / min (Engstad et al. 1992).

Counting of total leukocyte

In the blood samples, the total leukocyte were counted from sampled fish. For this purpose, a Neubauer counting chamber was used and method was followed according to by Schaperclaus et al. (1991). After collection of blood from fish, the certain amount of blood was combined with Natt–Herrick solution in a leukocyte pipe. The leukocytes were counted in duplicate samples twice from each fish.

Differential leukocyte count

The method of Steinhagen et al. (1990) and Schaperclaus et al. (1991) were followed with slight modification to obtain the percentage of cell types of leukocytes. The double stains; May–Grunwald and Giemsa were used to stain of the prepared blood smears. These staining procedure provided that the specific types of the leukocytes was seen under the light microscope. A total of 100 leukocytes in each slide were counted by using a Neubauer chamber. The percentage of the cell types were calculated.

Hematocrit level

During the sampling days, blood samples were collected from fish by using heparinized capillary tubes. From each fish, two heparinized capillary tubes full with blood were obtained. Then, the hematocrit centrifuge (worked at 10,500 g for 5 min) was used to read the hematocrit level of sampled blood. By following Steinhagen et al. 1990 and Schaperclaus et al. 1991, the percentage hematocrit value of blood samples was determined by using a special scale.

Statistics

All data were analyzed by one-way analysis of variance using the general linear model. Duncan's Multiple Range test was used to compare treatment means. Differrences were considered significant at the 0.05 probability level. All analysis was performed using the SPSS program.

Results

It has been suggested that hematological parameters are useful indicators for monitoring fish health and especially immune response. In this study, the mean number of NBT-positive cells in a microscopic field was presented in Table1. NBT-positive cells in the fish group which received 0.5 mg *M. comosum* were significantly increased ($P<0.05$) from the other groups on 21 and 28th days. This showed that giving dose increased phagocytic activity of neutrophils.

Total leukocyte counts in *M. comosum* (0.5 mg/fish) were determined significantly higher than other groups in 1 and 7 th days ($P<0.05$) (Table 1). While the first day after injection total leukocyte count was $0.69x10^5$/µl in control group fish blood, 0.5 mg/fish plant extract received fish groups showed highest cell count in 1st ($1.26x10^5$/µl) and 7th days ($1.14x10^5$/µl). The other samplings days the high values seen in the plant extract received fish groups compare to control group.

The percentage of neutrophils and monocytes also significantly ($P<0.05$) increased in blood of the 0.5 mg/fish plant extract received fish group (Table 2) compare to control and other group. The highest values of neutrophils and monocytes percentage were obtained in 0.5 mg/fish plant extract received fish group in each sampling days (Table 2). The following group of high values was determined on percentage of neutrophils and monocytes in blood of 2 mg/fish plant extract received fish group. The different concentration of plant extract injected fish groups expressed stimulated activity on neutrophils and monocyte cells.

The serum lysozyme activities values was highest levels at the 21st and 28th days in the 0.5 mg/fish plant extract received fish group hovewer, haematocrit levels in rainbow trout blood didn't change after injection of *M. comosum* geophyte plant ($P>0.05$) into all fish groups.

Our results revealed that, *M.comosum* stimulated nonspecific immune parameters in rainbow trout.

Table 1. Serum lysozyme activity (unit/ml), Hematocrit Level (%), NBT(+) neutrophil counts/microscopic field,Total leukocyte counts ($x10^5/\mu l$) in fish blood after i.p. injection of geophyte plant extract.

Days	Groups	Serum lysozyme activity (unit/ml)	Hematocrit level (%)	NBT(+) Neutrophil counts	Total leukocyte counts ($x10^5/\mu l$)
1	0.5mg	55.25±25.16^{a}	38.87±4.09^{ab}	12.29±7.67^{bc}	1.26± 0.24^{f}
	2mg	146.66±98.65^{abcd}	37.21±5.36^{ab}	15.13±8.63^{c}	0.76±0.17^{bcd}
	control	180±67.33^{abcd}	39.76±5.03^{ab}	4.24±3.37^{ab}	0.69±0.69^{bcd}
7	0.5mg	284±116.96^{d}	39.10±3.97^{ab}	4.61±4.18^{ab}	1.14±0.22^{ef}
	2mg	228±164.07^{cd}	41.05±2.57^{ab}	3.21±1.32^{ab}	0.70±0.14^{bcd}
	control	144±62.18^{abcd}	37.19±3.84 ^{ab}	3.38±1.95^{ab}	0.78±0.78^{bcd}
14	0.5mg	60±43.20^{a}	35.49±6.25^{a}	2.63±1.89^{a}	0.64±0.13^{abc}
	2mg	110±62.18^{abc}	39.64±5.70 ^{ab}	2.34±1.64^{a}	0.66±0.12^{abcd}
	control	125±66.08^{abc}	36.04±5.23^{a}	2.42±1.94^{a}	0.66±0.15^{abcd}
21	0.5mg	195±99.83^{abcd}	39.30±3.49 ^{ab}	14.53±12.88^{c}	0.54±0.05^{ab}
	2mg	220±160^{bcd}	43.13±2.82^{b}	2.82±1.16^{a}	0.81±0.16^{cd}
	control	164±58.99^{abcd}	38.17±3.07 ^{ab}	3.97±3.02^{ab}	0.91±0.29^{de}
28	0.5mg	152±109.17^{abcd}	41.01±3.75 ^{ab}	13.80±8.18^{c}	0.63±0.15^{abc}
	2mg	66.66±30.55^{ab}	35.04±1.61^{a}	7.19±6.99^{abc}	0.42±0.10^{a}
	control	120±20.00^{abc}	38.65±4.42 ^{ab}	4.16±2.09^{ab}	0.60±0.19^{abc}

Data are represented as mean ± SE (n = 10/group). Different letters represent the significant differences at $P < 0.05$

Table 2. The percentage of the leukocyte cells in fish blood after ip injection of geophyte plant extract.

Days	Groups	Leukocytes Percentage (%)		
		Lymphocyte	Monocyte	Neutrophil
1	0.5mg	83.6±2.07^{bcd}	7.8±1.48^{b}	8.6±2.19^{abc}
	2mg	70.8±6.41^{a}	6.8 ± 2.68^{b}	22.4±5.36^{d}
	control	87.2±3.11^{bcde}	2±0.7^{a}	10.80±3.19^{bc}
7	0.5mg	82.80±3.11^{bcd}	11.6±2.19^{c}	6±1.73^{ab}
	2mg	84±5.47^{bcd}	7.2±3.11^{b}	8.8±2.68^{abc}
	control	94.6±2.6^{ef}	1.8±0.83^{a}	3.8±2.49^{a}
14	0.5mg	85.40±3.91^{bcd}	6.8± 1.09^{b}	7.8±3.11^{abc}
	2mg	88.6±4.21^{cdef}	3.8±1.64^{a}	7.6±3.13^{ab}
	control	95±1.22^{ef}	1.8±0.44^{a}	3.4±0.54^{a}
21	0.5mg	79.40±10.52^{b}	6.8± 3.27^{b}	13.80±8.92^{c}
	2mg	89.2± 6.14^{def}	3.2 ±1.92^{a}	5.6±1.81^{ab}
	control	95.6± 1.81^{f}	1.6±0.54^{a}	2.8±1.48^{a}
28	0.5mg	70±5.61^{a}	7±2.44^{b}	23±2.44^{d}
	2mg	80.80±11.14^{bc}	3.2±1.92^{a}	10.2±7.52^{bc}
	control	93.6± 3.36^{ef}	1.5±0.57^{a}	3.4±1.67^{a}

Data are represented as mean ± SE (n = 10/group). Different letters represent the significant differences at $P < 0.05$

Discussion

Immunostimulants, influence growth performance and health condition in aquatic species by inducing a strong defence response against pathogens while minimizing the use of antibiotics. The immunostimulatory effects of plant extracts might vary by fish species, route of administration, dose, duration. Immunostimulants are critical in activate the immune responses capable of providing complete protection against certain pathogens.

The results of the present study indicate that there is a increasing number of NBT-positive cells after i.p.injection of *M. comosum* geophyte plant extract in rainbow trout (0.5mg/fish). Similarly, there have also been reported related results in various fish species, such as in gilthead sea bream (*Sparus aurata*) for *M. comosum* (Baba et al. 2014); in tilapia *Oreochromis mossambicus* for leaf extracts of *Ocimum sanctum* (Logambal et al. 2000) and *Tinospora cordifolia* (Sudhakaran et al. 2006); in rohu (*Labeo rohita*) for *Withania somnifera* (Sharma et al. 2010); nile tilapia (*Oreochromis niloticus*) for *Echinacea purpurea* and *Allium sativum* (Aly and Mohamed 2010); in common carp (*Cyprinus carpio*) for *Aegle marmelos* (Pratheepa et al. 2010).

Lysozyme is a fish defence element, which causes lysis of bacteria and activation of the complement system and phagocytes by acting as opsonin (Magnadottir 2006). Christybapita et al. (2007) was reported that enhanced serum lyzozim activity in *Oreochromis mossambicus* of *Eclipta alba* leaf extracts. Baba et al. (2014) also mentioned that the lysozyme activity of *Sparus aurata* serum well-enhanced after applying of i.p. injection of *M. comosum* plant extract. Similar elevated lysozyme activity in *Oreochromis mossambicus* serum was reported to acetone extract (1% w/w) from four medicinal plants (Bermuda grass, Cynodon dactylon; beal Aegle marmelos; winter cherry, *Withania somnifera* and ginger, *Zingiber officinale* (Immanuel et al. 2009). In addition, Awad et al. (2013) notify that they found increased levels of lysozyme in rainbow trout serum which fed with black cumin seed oil and nettle extract. In another study (Baba et al. 2016), all concentrations of oat extract-added (*Avena sativa*) diet fed fish serum lysozyme activity, significantly increased in the oat extract supplemented diet fed groups at all concentrations in *C. carpio*. However, in the present sdudy, all the doses of *M. comosum* did not increase the lysozyme activity in rainbow trout. Differences may be due to different fish species, plant extraction method, route of administration and time used in these studies.

In the present study, the percentage of neutrophils and monocytes were significantly elevated ($P<0.05$) that resulted in a significant increase in the total leukocyte counts in *M. comosum* (0.5mg/fish) applied fish. Similarly, Baba et al. (2014) also noted increased total leukocyte count, neutrophils and monocytes in *Sparus aurata* applied with *M. comosum* plant extract. In the other earlier study, the lymphocyte counts and total leukocyte counts also increased in *Oreochromis niloticus* given *Echinacea purpurea* supplemented diet with for 1 and 2 months feding period (Aly and Mohamed 2010).

In conclusion, *M. comosum* geophye plant extract which was given i.p. route into fish, improved and enhanced some of the nonspecific immune defense in rainbow trout. *M. comosum* could be suggested to be used for rainbow trout culture to strengthen the nonspecific immune system of fish. Thus, if it will be applied before outbreaks of disease, high numbers of mortalities might be avoided. Also, there is a need to further studies especially on the long-term feeding trials to determine the immunostimulant effects of this geophyte plant extract.

Acknowledgements

This work is a part of the research project number 104V126 financially supported by The Scientific and Technological Research Council of Turkey (TUBİTAK). This study was prensented in EAFP 2007 in Grado, Italy as oral presentation.

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