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The Effects of Injected Geophyte Plant (*Sternbergia candida)* Extract on the Hemato-Immunological Parameters in Gilthead Seabream, *Sparus aurata* (L. 1758)

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HIGHLIGHTS

- The plant extract show enhancement on non-specific immune response in gilthead seabream.
- Short term application of *Sternbergia candida* give a valuable result for fish farms.
- This study result obtained in natural environmental condition in a gilthead seabream farm.

Abstract: This study investigated effects of a geophyte plant extract (*Sternbergia candida*) on some hemato-immunological parameters in gilthead seabream, *Sparus aurata* (L. 1758). To serve this purpose, the plant was collected and extracted in ethanol solvent. A total 300 fish were used in experiment. Three groups were designed including control group, 0.5 mg/fish and 2 mg/fish and plant extract was given by intraperitoneal injection. Following injections on the 1st, 7th, 14th, 21st, 28th and 35th days, blood samples were collected from each group. Some of the hemato-immunological parameters in serum and blood samples such as, phagocytic activity (NBT (+) cells), total leukocyte level, lysozyme activity, total protein, hematocrit level and the differential leucocyte were examined. Present results show that intraperitoneal injection of *S. candida* plant extract increases some non-specific immune parameters. In the determination of the non-specific immune responses of the gilthead seabream, blood samples collected from 2mg/fish group indicated that maximum levels of pre-determined parameters were reached on days 7, 14, and 21 following the injection. According to these finding, administration of geophyte plant is suggested in seabream to improve non-specific immune response and health status.

Keywords: immunity; plant extract; *Sternbergia candida*; fish; injection.

INTRODUCTION

Gilthead seabream (*S. aurata* (L. 1758)) is an eminently marine species, which is widely common in aquaculture activity worldwide including Turkey. However, stocking density in fish farms has led to problems such as environmental damage, increased numbers of opportunistic microorganisms and stress conditions. Such adverse events may compromise the growth and health of fish and make animals more susceptible to infections and disease, causing significant economic losses [1]. For modern processes of aquaculture, prophylactic methods have become indispensable since significant progress has been made by stimulating the fish immune system through prophylactic methods [2]. Currently, the main prophylactic measures available for farmed fish include vaccination, probiotics and immunostimulant [3]. Throughout human history, a good diversity of herbs has been utilized around the world to make improvements on human health, help provide resistance against multiple infections or treat diseases [4]. In addition, they can be used as an alternative to chemotherapy and vaccination [5].

Natural products can be used as prevention against stress and infectious diseases as well as therapeutic and growth promoters [6]. The idea was to use natural products which are friendly to the environment to strengthen the immune system of fish [7, 8, 9, 10]. *S. candida* was chosen for this study because it is an endemic geophyte species and has not been tested on fish. *S. candida* is a significant geophyte plant. Plant extract is obtained from *S. candida* bulbous belonging to Amaryllidaceae family [11]. It wildly grows in Turkey, Africa, Asia and Europe [12]. All species of this family are known to have immunostimulant, antimicrobial, antiviral and antitumor activities also to enhance immune response [13, 14] but the activity of this plant have not yet been researched in fish species. In this sense, numerous efforts have been made to evaluate the immunostimulant effects of natural products in different fish species. It has been reported that several natural products, such as *Trigonella foenum graceum* [15, 16], *Nigella sativa* [17], *Excoecaria agallocha* [18], *Ducrasia anethifolia* [19], *Mentha piperita* [20], *Camellia sinensis* [21] and *Aloe vera* [22] enhance the non-specific immune response and disease resistance in various fish species.

It is important to underline the determination of hematological parameters for diagnosis of disease and abnormal situations. At same time, enhancement of fish immune system is necessary by using immunostimulant [23, 24, 25]. The using hematological analysis of blood in alive fish is important because it provides an evaluation of its health [26, 27, 28].

The aim of the present study was to determine the effects of injected *S. candida* geophyte plant extract on non-specific immune system in *S. aurata*. The potential use of this plant as a stimulant in fish immune system was investigated.

MATERIAL AND METHODS

Plant extract

S. candida was obtained from the natural area in Mugla, Turkey. The bulb parts of the plant were washed with distilled water then cut into small pieces. The bulb pieces were air dried in a shady area for two weeks before being grounded to powder (200-250 g dry weight) and extracted with 500 mL balloon flasks of ethyl alcohol for 24 h according to the methods of Lee and coauthors [29]. The extract was filtered with Whatman filter paper (0.45 µm). This process was repeated three times. The solvent was evaporated from the crude extract using lyophilizator and extract kept at 4 °C in dark bottles until further use.

Fish and trial design

The marine fish, gilthead seabream, *S. aurata* (112.00 ± 1.5 g) were provided from a commercial fish farm (Mugla-Bodrum in the facilities of Kilic Seafish Import Export Corporation at Guvercinlik, Turkey). The study was conducted by following Mugla Sıtkı Koçman University approved ethical rules. The fish were maintained in 1500 L aerated fiberglass tanks. The water quality was recorded by using multiprobe (YSI 556 MPS) and measured as follows: temperature 23 ^{o}C, pH 7.6 salinity % o 37 and dissolved oxygen 7.5 mg/L. A total of 300 fish were used in the experiment. After two weeks acclimatization periods, fish were randomly divided into nine fiberglass tanks in 3 duplicate groups at a stocking density of 50 fish/tank. Sterile phosphate buffered saline (PBS) soluble geophyte plant extract (*S. candida*) was applied to each fish by intraperitoneal injection method. The first and second group inoculated 0.1 mL of the geophyte plant extract in different concentrations (first group: 0.5 mg/fish; second group: 2mg/fish) by injection method. The control group was inoculated only PBS in the same volume (0.1 mL). Sampling was done on the 1st, 7th, 14th, 21st, 28th and 35th days after intraperitoneal injection.

Serum and Blood Cells

Five gilthead seabreams from each individual tank were randomly collected and anaesthetized with phenoxyethanol. The blood samples were taken from caudal vein. Some of the blood was put into an Eppendorf tube for analysis. The other portion of blood samples were kept at 4 °C for overnight and later the serum was collected after centrifugation (3500 g, 5 min, 4 °C). Serum samples were stored at -80 °C until use for other analysis.

Nitro blue tetrazolium (NBT) assay

Respiratory burst activity was determined according to the method described by Anderson and coauthors [30]. Briefly, NBT suspension (0.2%) was prepared with sterile saline solution (0.85%). Thereafter, 50 µL of blood was dropped onto a coverslip and incubated for 30 min at 25 °C. The details of each stages were described in our previous paper [31].

Lysozyme activity

The serum lysozyme activity was determined in turbidimetric assay with minor modification [32, 33]. In brief, 25 µL of fish serum were added 175 µL *Micrococcus lysodeikticus* suspensions in duplicate well of a microplate. The reaction incubated at 22 °C and absorbance 490 nm was carried out after 0.5 and 4.5 min using an ELISA microplate reader. A unit of lysozyme activity was defined as a decrease in absorbance of 0.001/min.

Total protein

The Bradford assay for total protein was measured according to Bradford [34]. The reaction was carried out in a 96 well plate. The absorbance was read at 595 nm by a multi-scan microplate reader. The standard solution was prepared using bovine serum albumin. Serum total protein concentration were expressed as mg/mL.

Hematological parameters

Hematocrit (%), total white blood cells (WBC, 10^3 cell/mm^3) count and differential leukocyte count (monocyte, neutrophil, eosinophil, lymphocyte (%)) was calculated as hematological parameters. Percent hematocrit level was determined with microcentrifuge technique, using heparinized capillary tubes. For WBC count Natt–Herrick solution method was used. Total leukocyte amounts were counted using Neubauer counting chamber. Differential leukocyte count was made in peripheral blood smears stained by May-Grunwald and Giemsa stains and percentages of leukocyte types were calculated [35, 36].

Statistical analysis

Effects of plant extract on hemato-immunological parameters were expressed as the arithmetic means standard error (SE) by using Tukey's pairwise multiple comparison test of variance (ANOVA). Differences were considered significant at $P < 0.05$.

RESULTS

NBT-Positive cell count

NBT positive cell counts of samples determined that there were differences (Figure 1) among the trial groups and the control group on certain days ($P < 0.05$). The respiratory burst activity significantly increased in all the experiment groups during the trial period. The highest activity was recorded at days 7th, 14th and 21st in the 2 mg/fish group.

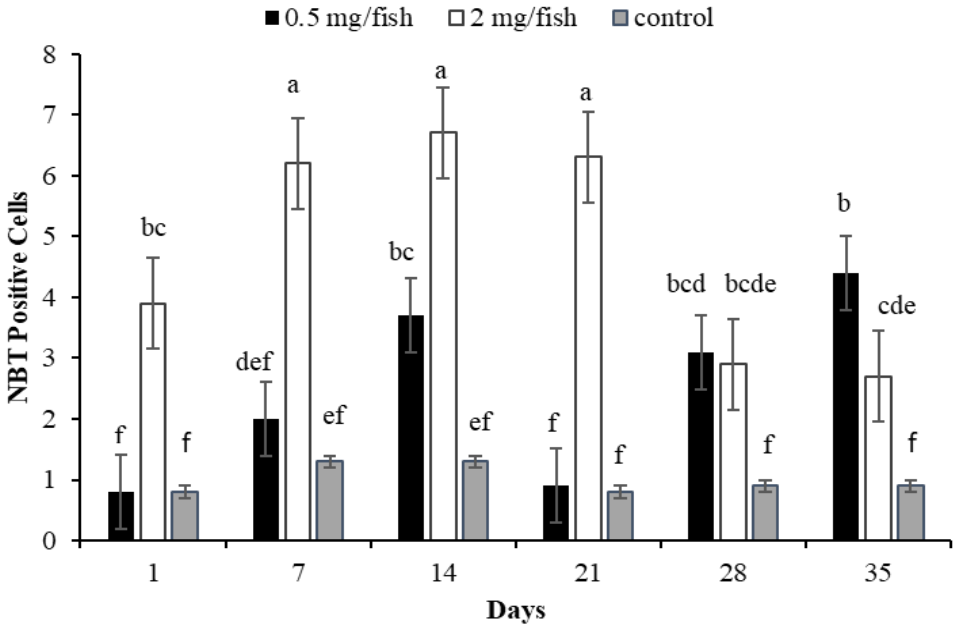

Figure 1. Effects of plant extract on the number of NBT-positive cells of seabream. Vertical lines are mean± SEM [SEM.: standard error mean]. Different letters represent significant differences at P < 0.05.

Lysozyme activity

The effect of plant extract injected fish on the lysozyme activity in serum is exhibited in Figure 2. The serum lysozyme activity was detected to be higher in 2 mg/fish concentration of plant extract injected fish serum than in 0.5 mg/fish and the control group during the experiment time. (P < 0.05). Among the concentrations and the days tested, the 2 mg/fish showed the highest lysozyme activity at days 7th, 14th and 21st.

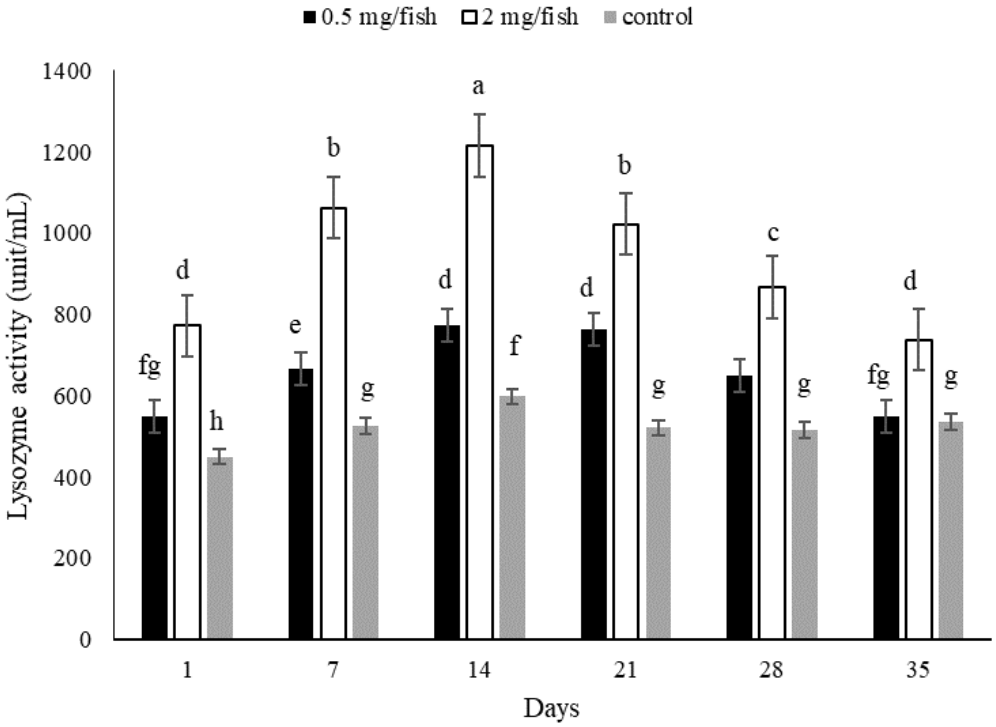

Figure 2. Changes of lysozyme activity in serum of seabream. Vertical lines are mean± SEM [SEM.: standard error mean]. Different letters represent significant differences at P < 0.05.

Total protein amount

Total serum protein amount was not significantly increased (P > 0.05) in injected fish (0.5 and 2 mg/fish) with plant extract as compared to control for experimental period (Figure 3).

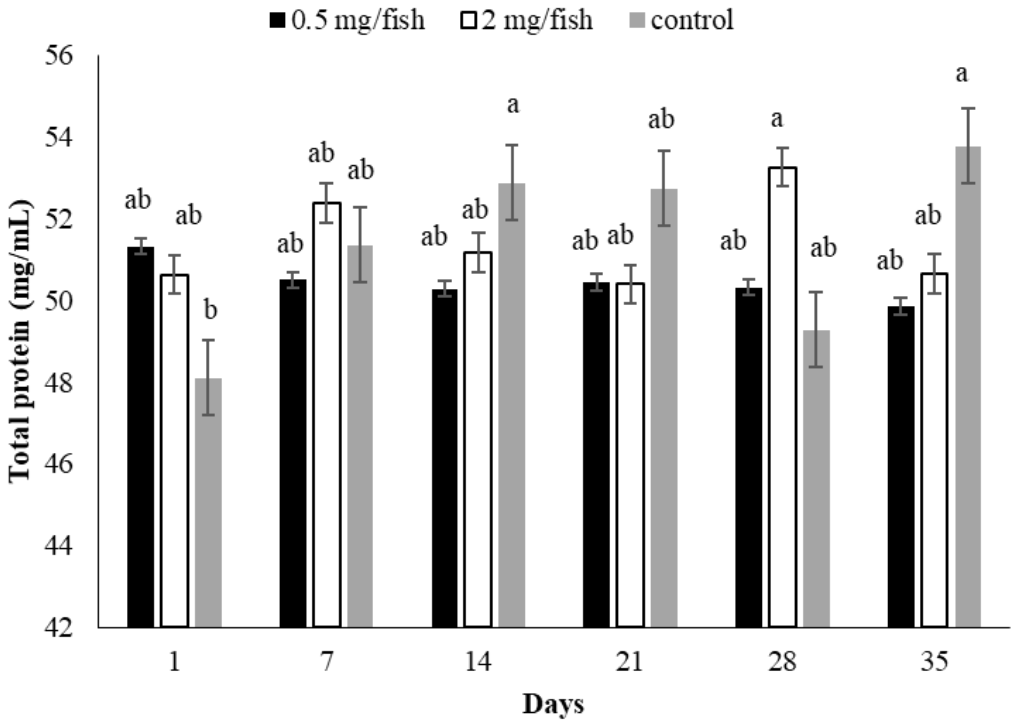

Figure 3. Effects of plant extract on total protein level in serum of sea bass. Vertical lines are mean± SEM [SEM:standard error mean]. Different letters represent significant differences at P < 0.05.

Hematological parameters

WBC number, especially those injected 2 % concentration which showed increase (P < 0.05) compared to the values found in fish from the control group. On other days, it is detected that the injected plant extract had no effect on WBC of blood samples in gilthead seabream (P > 0.05). No significant changes (P > 0.05) were determined in hematocrit level (Table 1) among experimental groups compared to the control group. Besides phagocytic blood cells (monocytes, neutrophils, and eosinophils) were significantly increased (P < 0.05) in all experimental groups compared to those in the control (Table 1). The lymphocyte amount did not show much variation between the control and experimental groups. According to the recorded data in this study experimental groups showed the highest effect of monocytes, neutrophils and eosinophils on 7th, 14th and 21st days.

Table 1. The percentage of lymphocyte, monocyte, neutrophil, and eosinophil cells in blood samples of seabream injected plant extract.

Days	Dose	WBC	M	N	E	L	Hct
1	0.5	32.14±4.08^{h}	4.5±0.48^{def}	5.10±0.49^{bc}	7.60±0.78^{de}	82.80±0.99^{ef}	33.50±1.13^{def}
	2	63.12±4.27^{fg}	5.90±0.52^{bc}	5.70±0.13^{b}	7.60±0.45^{de}	80.80±0.37^{ef}	36.80±1.33^{cde}
	C	52.02±3.89^{g}	4.20±0.50^{def}	3.10±0.37^{e}	3.40±0.35^{g}	89.30±0.92^{abc}	28.90±1.98^{f}
7	0.5	87.02±5.83^{de}	4.10±0.32^{ef}	5.10±0.48^{bc}	7.30±1.34^{de}	83.50±1.04^{de}	35.00±1.36^{cde}
	2	90.77±5.25^{cde}	5.50±0.69^{cd}	4.90±0.28^{bc}	8.00±0.78^{de}	81.60±0.87^{ef}	40.10±0.61^{bc}
	C	82.49±3.61^{e}	2.70±0.27^{ghı}	2.70±0.22^{ef}	3.40±0.19^{g}	91.40±0.62^{a}	36.60±1.83^{cde}
14	0.5	107.65±6.13^{bc}	7.10±0.60^{ab}	6.10±0.92^{b}	6.40±0.82^{ef}	80.40±1.25^{ef}	36.70± 1.45^{cde}
	2	113.74±5.63^{b}	8.10±0.24^{a}	7.80±0.50^{a}	9.20±0.64^{d}	74.90±1.29 ^{g}	35.40±0.78^{cde}
	C	119.21±4.63^{b}	3.40±0.33^{ghı}	3.20±0.24^{e}	4.60±0.26^{fg}	88.80±0.64^{abc}	38.00±1.45^{bcde}
21	0.5	139.83±4.28^{a}	5.25±0.73^{cde}	4.45±0.27^{cd}	25.10±1.56^{a}	67.20±1.02^{h}	42.20±1.89^{ab}
	2	115.77±4.83^{b}	6.10±0.52^{bc}	5.90±0.54^{b}	23.05±1.89^{a}	64.95±1.64^{h}	39.40±3.96^{bc}
	C	115.93±3.24^{b}	2.60±0.33^{ghı}	3.60±0.49^{de}	7.80±0.24^{de}	86.40±0.80^{cd}	38.80±0.54^{bc}
28	0.5	86.08±4.05^{de}	3.20±0.38^{fgh}	1.80±0.24^{fg}	14.80±0.53^{b}	80.20±1.10^{ef}	42.10±0.46^{ab}
	2	93.30±4.20^{cde}	5.20±0.24^{cde}	3.40±0.16^{de}	11.60±0.45^{c}	79.80±0.24^{f}	46.70±1.76^{a}
	C	75.77±4.94^{ef}	2.20±0.24^{ghı}	1.60±0.16^{fg}	8.60±0.45^{de}	87.60±0.45^{bc}	38.20±0.85^{bcde}
35	0.5	102.96±5.06^{bcd}	1.80±0.24^{ı}	1.20±0.13^{g}	6.00±0.29^{ef}	91.00±0.47^{ab}	33.20±1.34^{ef}
	2	107.02±4.74^{bc}	2.20±0.24^{ghı}	1.40±0.16^{g}	7.00±0.36^{def}	89.60±0.68^{abc}	37.30±1.03^{bcde}
	C	63.70±3.47^{fg}	2.00±0.29^{hı}	1.40±0.16^{g}	6.20±0.38^{ef}	90.80±0.67^{ab}	38.50±1.16^{bcd}

Means having different superscript letters in same column are significantly different from each other $P < 0.05$. (C: control, WBC: white blode cells (10^3 cell/mm^3), M: monocyte (%), N: Neutrophil (%), E: eosinophil (%), L: lymphocyte (%), Hct: hematocrit (%)).

DISCUSSION

The innate immune system components take place on the first line of fish defence against various pathogens in fresh and marine environmental conditions. The enhancement of the system is important for fish health [37, 38]. The application of herbs could enhance the immune mechanism of fish against all kind of pathogens in aquaculture [39, 40, 41].

This research explored the possible effect of *S. candida* geophyte plant extract intraperitoneally injected with different concentrations on the non-specific immune system in *S. aurata*. Our research detected different degree of effectiveness on immunological and hematological parameters in seabream, *S. aurata* within a 35 days experimental period. Respiratory burst activity is one of important defense mechanism of innate immunity [42]. In the present study, we detected increase in the number NBT positive cells. NBT positive cell numbers reached the maximum level on the 7th, 14th and 21st days in the 2 mg/fish experiment group. This is supported by the works of Anderson and coauthors [30], Anderson and Jeney [43], Chen and coauthors [44] in that NBT-positive cell numbers to increase in fish which were given immunostimulants by injection. Similar results were obtained in different fish species, such as *Oreochromis niloticus* [18], *Mugil cephalus* [21], *Epinephelus coioides* [45], *S. aurata* [16, 31]. Also, respiratory burst activity was significantly higher in seabream fish fed diets supplements with 0.1 % and 1% dihydroquercetin obtained from deodar (*Cedrus deodora*) compared to the control [46]. In fish, lysozyme is an important bacteriolytic enzyme against pathogen invasions [47]. Our results revealed an increase in lysozyme activity in experimental groups injected with plant extract for especially certain days. Feeding *O. niloticus* with *Excoecaria agallocha* for 28 days led to significant in serum lysozyme activity [18]. Kakoolaki and coauthors [21] observed increases in lysozyme activity in *Mugil cephalus* fed with different doses of *Camellia sinensis*. Similarly, feeding tilapia with different concentration *Aloe vera* led to an increase in lysozyme activity compared to control group [22]. Moreover, Bilen and coauthors [48] documented an increased activity of lysozyme in rainbow trout fed with various concentration *Pleurotus ostreatus* and *Urtica dioica.* Similarly, elevated lysozyme activity was noted on

Oncorhynchus mykiss [20], *Salmo trutta caspius* [49], *Oreochromis niloticus*, *Oreochromis aureus* [50], *Cyprinus carpio* [51].

The principle factor that accounts for the fluctuations in the plasmatic volume is the total amount of protein in the serum as a result of stressors [52]. This study exhibited that two concentration of *S. candida* plant extract did not alter total protein level compared to control group. In the line with this study, Baba and coauthors [31] reported that plant extract (*Muscari comosum*) did not change the serum total protein amount in *S. aurata.* Similarly, Dadras and coauthors [53] showed that dietary supplementation with two medicinal plant no alter the protein amount in the seum of *Huso huso*. Also, Vazirzadeh and coauthors [19] documented that *Ducrasia anethifolia* essential oil have no effects on serum protein level in *Oncorhynchus mykiss.*

In the field of aquaculture, haematological parameters are utilized as common signifiers for fish health conditions such as stress or disease [54]. Besides, in order to find out the impact of the prospective immunostimulants on the fish immune system, each of the parameters is investigated [55]. The role of white blood cell in innate immunity is well established. It constitutes the primary line of defense. As its numbers increase in parallel to other immunological parameters in the fish, it is used to determine their health status [56]. Our data showed that WBC of plant extract injected fish with 2 % concentration significantly increased after 28 th days. On other days, no difference we observed in number of WBC compared to control group. The obtained results agree with those obtained in common carp [42], rainbow trout [19, 20, 58], juvenile beluga [53], Caspian brown trout [49], seabream [15, 31]. The present study, it was observed that the monocyte, neutrophil and eosinophil counts in blood samples were increased on the fish injected with *S. candida* plant extract. It has been determined that the leukocyte cells in blood reached maximum level on the 7th, 14th and 21st in experimental groups (0.5 and 2 mg/fish). Overlay, hematocrit values were observed demonstrate any significant difference in all experiment groups. The hematocrit level of the fish blood is not negatively impacted by the plant extracts. In parallel with our results, hematocrit levels *O. mykiss* exhibited no alters after feeding with *Ducrosia anethifolia* essential oil [19]. Also, Nya and Austin [58] documented that feeding rainbow trout with garlic did not change the hematocrit level.

It is concluded that injected *S. candida* geophyte plant extract at the concentration especially 2 mg/fish enhanced the non-specific immune system. In the light of the data obtained, the *S. candida* geophyte plant extract can be added to the feed and applied to fish under aquaculture conditions. Nevertheless, future studies are needed to determine the effects of *S. candida* geophyte plant extract on the other cultured fish species.

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