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Effects of dietary garlic (*Allium sativum*) extract on survival rate, blood and immune parameters changes and disease resistance of Common carp (*Cyprinus carpio carpio* Linnaeus, 1758) against Spring Viremia of Carp (SVC)

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Abstract

This study evaluated the influence of dietary garlic (*Allium sativum*) extract on survival rate and changes in blood and immune parameters of Common carp *(Cyprinus carpio)*, fed with two different concentrations of aqueous garlic extract after exposure to spring viremia of carp virus (SVCV). The experiments have executed in two steps: Firstly, 270 fingerlings of Common carp with an average weight of 15±3.4 g have cultivated in 18 aquaria with 15 liters of water. Fish were fed with 1 and 5 g garlic extract kg^{-1} basal diet for 8 weeks. In the second step, 240 fingerlings have been randomly divided into 8 groups (A: negative control; B: virus control; C and D: extract controls and E-H: treatment groups) with 3 replicates in each group include of 10 fish in each replicate, and the fish have been exposed to SVCV for 4 weeks. The mortality rate, blood and immune parameters and virus isolation have been determined at the end of experiments. The results have showed that the survival rate of the Common carp fed with 1 and 5 g garlic extract kg^{-1} basal diet (E-H groups) had a significant increase compared to the control groups (p<0.05) and RBC, hemoglobin, hematocrit, MCH, MCHC had Significant increase compared to the virus control group (B) (p<0.05). WBC in groups D, E, F and G in comparison to control group A and B and lymphocyte in groups D, E and F compared to control group B had a Significant increase (p<0.05). Also, the Common carp fed with two concentrations of garlic extract increased lysozyme and IgM in groups C-H compared to group A (p<0.05). Finally, cell culture and RT-PCR have been applied for detection of SVC virus from fish samples. The results have been indicated that addition of garlic extract (Especially 5 g kg^{-1}) in fish dietary has led to a higher immunity and survival rate of common carp exposed to SVCV.

Keywords: Garlic extract, *Cyprinus carpio*, Survival rate, Blood and immune parameters, SVCV

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Introduction

The use of herbal immune-stimulants in the aquaculture, to increase the immune system activities and fish resistance against various diseases has spread worldwide. Many studies have shown that immune-stimulants of plants enhance immune system, as well as increase or reduce fish losses against a variety infections (Nya and Austin, 2009; Yin *et al*., 2009; Ardo, 2013; Haghighi and Sharif Rohani, 2013; Sharif Rohani *et al*, 2013; Bazari Moghaddam *et al*., 2017; Yuvaraj and Arul, 2017; Zadmajid and Mohammadi, 2017). Garlic (*Allium sativum*) of the Alliaceae family is one of the most important native plants in Iran and has significant therapeutic value (Hussein *et al*., 2013). Garlic plant has several anti-microbial, anti-carcinogenic, anti-fungal and anti-stress properties and also known as a factor in improving nutritional indices, immune and growth stimulants, antioxidants, and also balancing blood pressure (Kumar and Berwal, 1998; Fazlolahzadeh *et al*., 2011). Among the most important garlic compounds, allicin, phosphoric compounds, alkaline enzymes, peroxidase, ajuvin, citral and granulated are mentioned. Some studies have shown that garlic consumption increases the production of cytokines, the activity of macrophages and lymphocytes, and ultimately improves and stimulates the immune system (Khodadadi *et al*., 2013). Garlic is rich in minerals (iron, iodine, sodium, potassium and phosphorus), and useful vitamins (A and C) for the body of the fish (Farahi *et al*., 2010). The presence of beneficial compounds in garlic, especially allicin, has introduced this plant as a strong antimicrobial and immune-growth enhancer. The Common carp (*Cyprinus carpio*) is considered to be the most important and largest breeding fish for human nutrition (Sanders *et al*., 2003). In recent years, Common carp is becoming more important cultured fish in the world. With the development of Common carp culture, effects of feeds with supplement herbal extracts on the balance of the growth and immune responses are highly needed. Some previous studies have focused on the effects of garlic extract on the immunity of Common carp (Shalaby *et al*., 2006; Ndong and Fall, 2011; Nwabueze, 2012; Talpur and Ikhwanuddin, 2012). Spring Viremia of Carp is a highly lethal and contagious disease in the Cyprinidea family and their related species. The most important symptoms of this disease include subcutaneous, hemorrhage, abdominal swelling, exophthalmia, imbalance and death of fish. Mortality rates are sometimes reported up to 70% (OIE, 2018). The purpose of this study is to evaluate the effects of garlic extract on survival rate and changes in blood and immunity parameters of Common carp, fed with two different concentrations of garlic extract after exposure to spring viremia of carp virus (SVCV).

Materials and methods

Preparation of aqueous garlic extract

Fresh garlics have been collected from one of the farms in the province of Gilan, located in Astaneh Ashrafieh

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then have been left for 30 minutes after cutting (the enzyme alliinase in the garlic converts alliin into allicin), then the garlic have mixed with phosphate saline buffer (pH=7.2 and PBS) in a blender in equilibrium proportion and passed through multi-layer sterile gas then have been centrifuged (model: 5810R-Eppendrof) at 3400 g for 10 min at 4 °C, Finally, the supernatants have been sterilized thru 0.22 µm filter (model: Millipore) and stored at 4°C (Ghazanfari *et al*., 2006). High-performance liquid chromatography (HPLC) has used to determine the amount of active ingredient in the garlic extract (Allicin) (Model: 1525 with detector UV-VI, American Waters).

Supplementation of the normal diet with Garlic extract

The basic diet containing 34.9% protein, 12.8% fat, 11.2% ash, 10% moisture and 31.1% total carbohydrate have been prepared from Roohin Company. Aqueous garlic extract have been added to the basic diet at 1 and 5 g kg^{-1}. The mentioned amounts have been mixed with 50 g of the ration and then added to the rest of the diet and mixed with an electric mixer for 20 minutes until homogenized. After adding some water to the composition and forming the dough, the meat grinder have been used to turn the food into cylindrical pellets. Finally, the pellets dried in a dryer at 30 °C for 24 hours. The diameter of the pellets was 2 mm while their length was 4 mm. The pellets were then packed and kept at 4 °C (Luo *et al*., 2010).

Fish and experimental conditions

This research has been conducted at the fish disease laboratory in Inland water aquaculture research center. 270 Common carp fingerlings with an average weight of 15±3.4 g and a mean length of 9.88±0.78 cm have been obtained from a private fish farm in Rasht. These fish have been introduced to 18 aquariums containing 15 fish in each aquarium. After 14 days adaptation, research has executed in a completely randomized design including 3 treatments with 3 replications containing control (no garlic extract), 1 and 5 g garlic extract kg^{-1} basal diet and fingerlings have been fed three times per day for 8 weeks. Mean temperature, dissolved oxygen and pH during culture period were 17±1.7 °C, 7.2±0.47 mg L^{-1} and 7.51±0.81, respectively. After 8 weeks, the fingerlings have been challenged to the spring viremia of carp virus (SVCV) (isolate 56/70, Accession No. Z37505.1) (Stone *et al*., 2003). For this step, 240 Common carp have been transferred to 24 aquariums equipped with aeration. At this stage, the fish have been divided into treatment and control groups (with three replicates) in the 4 weeks period according to the following model: Group (A) - Fish without extract and virus, Group (B) - Fish received the virus, Group (C) - Fish received garlic extract (1 g kg^{-1}), Group (D) - Fish received garlic extract (5 g kg^{-1}basal diet), Group (E) - Fish received garlic extract (1 g kg^{-1} basal diet) and exposed to a virus that has been simultaneously adjacent with the extract. Group (F) - Fish received garlic

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extract (1 g kg^{-1}basal diet) and exposed to a virus that has been adjacent to the extract four hours ago, Group (G) - Fish that had garlic extract (5 g kg^{-1}basal diet) treated with the virus that was simultaneously exposed to the extract. Group (H) - Fish received garlic extract (5 g kg^{-1}basal diet) and has been exposed to a virus which has been adjacent to the extract for four hours ago.

Blood sampling

At the end of the 4 weeks, blood samples have been collected from the surviving fish. From each group, 9 fish have randomly assigned. Feeding has deprived 24 hours before blood collection, and then blood samples have been taken using a 2 ml syringe. For blood sampling, anaesthetics have not used due to the possibility of affecting blood parameters (Torrecillas *et al*., 2011). 1 ml of serum collected in eppendorf tubes without an anticoagulant (heparin) and 0.5 ml in an eppendorf tubes containing heparin. Then samples have been centrifuged (model: 5810R-Eppendorf) using centrifugation at 3000 rpm for 10 minutes. The serum has separated and stored at -80 °C. Blood parameters include RBC, hematocrit, hemoglobin, WBC, differential WBC including Lymphocytes, Eosinophils, Neutrophils and Monocytes, MCV, MCH and MCHC which were measured using the standard methods.

Hematological assay

Blood samples have analyzed following standard methods adopted in fish hematology. The total red blood cell counts (RBC×10^6 ml^{-1}) have been determined in a 1:200 dilution of the blood sample in Ress solution and total white blood cell counts (WBC×10^3 ml^{-1}) in a 1:20 dilution of the blood sample with a Neubauer hemocytometer. The hematocrit (Hct) have determined by using micro hematocrit method (Houston, 1990; Klontz, 1994). The hemoglobin (Hb, g dl^{-1}) concentrations have determined by the cyanmethemoglobin method (Klontz, 1994) using a hemoglobin reagent set (Pars Azmun Diagnostics). All the values of red blood cell indices, the mean values of cell hemoglobin (MCH pg), cell hemoglobin concentration (MCHC %), and cell hemoglobin volume (MCV fl) have been calculated according to Wintrobe formulae (Anderson and Klontz, 1965). The differential leukocytes count has carried out using blood smears stained with Wright-Giemsa (Klontz, 1994).

Immunological assay

The turbidimetric assay for lysozyme has carried out according to Sahoo *et al*. (2008). The nephelometric method for Immunoglobulin M (IgM) has been recommended by Yeh *et al*. (2008). In this method, the IgM contained in the blood serum sample with a polyclonal anti-IgM antibody forms a complex and causes clouding of the solution. The monochromatic light photomultiplier scans the solution in wavelengths between 400-840 nm, which after the collision the antibody complex and the antigen are dispersed, and the degree of

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differentiation is proportional to the amount of IgM.

Mortality rate

At the end of the experiment, fish losses in treatment and control groups have counted and subsequently the mortality rate of fish fed with garlic extract has calculated in the face of SVCV and fish sampling was done to isolate and detect SVCV in tissue samples. Samples have been taken from the gill and kidneys of fish (OIE, 2018). Separated samples have transferred to -80 ºC for virus isolation and cell culture experiments.

Cell culture

The EPC cells have cultured by using Eagle's Minimum Essential Medium (EMEM), supplemented with 10% fetal bovine serum (FBS) in a 25 cm^2 tissue culture flask (Orange scientific Co., Ltd., Denmark) seeded in 24 well plates. Fish kidney and gill samples have been homogenized in nine volumes of EMEM centrifuged and filtered through a 0.45 mm membrane. A 150 ml of this supernatant has inoculated to the 24h EPC cells. After standing at room temperature for 60 min, the plate has washed up once with HBSS, supplied with EMEM (5% FBS), and incubated at 15 °C The inoculated cells have been observed under inverted microscope for 10 days detect viral CPE (OIE, 2018).

Total RNA extraction and RT-PCR

For confirmation of virus isolation, RT-PCR has performed on tissue samples obtained from dead and survived fish. Total RNA extraction has performed using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Briefly, tissues from dead and survived fish from different virus exposure models have been pooled separately and 30 mg of fish tissue samples have been transferred to 1.5 ml sterile micro tubes, then 600 ml RLT buffer was added. The cell lysates have been centrifuged at 8000 g for 3 min at 4 ºC. Total RNA has precipitated by ethanol and purified using RNeasy spin columns. The RT-PCR assay has performed using the kit Qiagen One-Step RT-PCR. Reverse transcription reagents [5 mL of 5_ RT-PCR buffer, 1 ml dNTPs mix, 1 ml one step RT-PCR enzyme mix and 13 ml RNase free distilled water] as well as 2 ml of forward and reverse primers were added to 3 ml RNA samples to make a final reaction volume of 25 ml in each case. The primer pairs for RT-PCR have been derived from nucleotides 814–835 and 1262–1283 of the G gene (Koutna *et al*., 2003). The reaction mix has incubated at 50 °C for 30 min followed by 15 min at 94 °C. Then, 35 cycles of PCR, denaturation for 30 s at 94 °C, annealing for 30 s at 50 °C and polymerization at 72 °C for 1 min, has been conducted. The polymerization has been concluded by an extension period of 10 min at 72 °C. Then 470 bp RT-PCR product was visible after electrophoresis in a 1% agarose gel containing ethidium bromide (0.2 mg ml^{-1}) and under UV trans illumination.

Statistical analysis

The present study has been conducted in a completely randomized design. The

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data have been normalized by Kolmogorov-Smirnov test and homogeneity test has performed by Levene test. In the case of homogeneity of data, one way ANOVA has been used to compare the mean of nutritional treatments and the Duncan test. Non-parametric Kruskal-Wallis test has used for non-homogeneous data. The significance of the groups has determined using the Mann-Whitney test. The SPSS statistical software, the 19th edition, has been used for data analysis.

Results

The results of HPLC on the active ingredients of garlic have showed that the amount of Allicin measured in garlic extract of 200 mg g^{-1}. After exposing the fish to SVCV and completing the 4-week period, the results of mortality and survival rate as well as the results of blood and immune parameters have presented as follows:

Mortality rate

According to the table 1, at the beginning of the third week, the first mortalities have observed in the positive control treatment of the virus (group B). After statistical analysis, there was a significant difference between group B (virus control group) and each of the group C, D and A (which had not been exposed to the virus and all fish survived) separately (p<0.05). Diseased fish usually appeared darkness. Typical clinical signs include exophthalmia, pale gills, hemorrhage on the skin, base of the fins and the vent, abdominal distension or dropsy and a protruding vent (anus), often with trailing mucoid fecal casts (Fig. 1). The low mortality rate has observed in treatment E and F. There were the lowest mortalities in G and H groups and a significant difference in the survival rate of fish in group G, H, E and F compared to group B (p<0.05). There was no statistically significant difference in survival rate of fish between group G and H and between Group E and F (p>0.05).

Table 1: The Mortality rate of Common carp fed with different levels of garlic extract for two months and then exposed to SVCV for 4-weeks.

Groups	Initial number of fish	1-week (dead fish)	2-week (dead fish)	3-week (dead fish)	4-week (dead fish)	Sum of dead fish for 4 weeks	Mortality rate (%)	Survivability rate (%)
A	30	0	0	0	0	0	0^{c}	100^{c}
B	30	0	0	7.2	12.6	19.8	66^{a}	34^{a}
C	30	0	0	0	0	0	0^{c}	100^{c}
D	30	0	0	0	0	0	0^{c}	100^{c}
E	30	0	0	5.8	4.5	10.3	34^{b}	66^{b}
F	30	0	0	4.5	4.3	8.8	29^{b}	71^{b}
G	30	0	0	4	3.7	7.7	26^{b}	74^{b}
H	30	0	0	3.1	3	6.1	20^{b}	80^{b}

Data in columns are expressed as mean±SD,
Differences in Latin letters in each column shows a significant difference (p<0.05).

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(a) (b)

Figure 1: Some clinical signs have been observed in dead fish due to SVCV infection (a, b).

Evaluation of blood and immune system parameters

According to the Table 2, comparison between the fish fed with garlic extract without virus (both concentrations), i.e. groups C and D with group A, it has seen that the levels of hematocrit, hemoglobin, RBC, WBC, MCV, MCH, MCHC, lymphocytes and neutrophils increased while monocytes and eosinophils levels have been decreased. In the comparison between group B with group A, significant decrease has observed in the hematocrit, hemoglobin, RBC, MCH, MCV, MCHC, and lymphocytes (p<0.05), while at the same time, a significant increase has observed in the WBC, monocytes, neutrophils and eosinophils (p<0.05). In comparison among groups E, F, G and H with group A, hematocrit, hemoglobin, RBC, MCV, MCH, MCHC and lymphocyte decreased (p>0.05), lymphocytes in groups E and F and MCV in group E had significant decrease compared with group A, while WBC, monocytes, neutrophils and eosinophils increased (p>0.05), WBC in groups E, F and G; monocytes in groups E, F and G and neutrophils in group E had significant increase comparing with group A. In comparing between groups E and F, group F had higher levels of hematocrit, hemoglobin, RBC, MCV, MCH, MCHC and lymphocytes (p>0.05), MCV in group E had significant difference with group F, but WBC, monocytes, neutrophils and eosinophils in Group F was lower than group E (p>0.05), of course neutrophils had significant increase in group E. In comparison between groups E and G, group G had higher hematocrit, MCV, MCH, MCHC and lymphocytes levels, and a higher number of hemoglobin and RBC (p>0.05), MCV in group E had significant difference with group G, but the number WBC, monocytes, neutrophils and eosinophils was lower than that of group E (p>0.05), neutrophils had significant difference in group E. comparison between groups F and H, It showed that group H had higher hematocrit, MCV, MCH, MCHC and hemoglobin and RBC and lymphocytes levels (p>0.05), but WBC, neutrophils, monocytes and eosinophils were lower (p>0.05), especially WBC and monocytes in group H had significant difference with group F. According to table 3, in the comparison

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between groups C and D with group A showed that the level of lysozyme and IgM was significantly higher in group D compared to A and C (p<0.05). In comparison between group B and group A, it has found that the level of lysozyme and IgM in group B was higher than group A (p<0.05). The groups of E, F, G and H were compared separately with group A, four groups had higher levels of lysozyme and IgM (p<0.05). Groups E, F, G and H were compared with group B. Four groups had higher levels of lysozyme and IgM (p>0.05). The comparison among groups E, F, G and H, showed there was no difference between the groups (p>0.05).

Table 2: The hematological parameters of Common carp fed with different levels of garlic extract for two months then exposed with SVCV for 4-weeks (mean ±SD).

Blood parameters	A	B	C	D	E	F	G	H
Hct (%)	35 ± 0.1^{b}	26.2 ± 2.1^{a}	36.2 ± 1.1^{b}	37± 2.3^{b}	32.8 ±1.2^{b}	32.9 ± 6.7^{b}	33.1 ± 5.1^{b}	33.5 ± 7.2^{b}
Hb (g dL^{-1})	7.4 ± 0.05^{b}	3.1^{a} ± 6.4	7.5 ± 4.1^{b}	7.6 ± 0.21^{b}	6.9 ± 3.1^{b}	6.9 ± 8.6^{b}	7.0 ± 1.2^{b}	7.1 ± 3.4^{b}
RBC (10^{6} ml^{-1})	0.79 ± 0.05^{b}	0.1^{a} ± 0.69	0.80 ± 8.3^{b}	0.81 ± 5.1^{b}	0.74 ± 3.3^{b}	0.74 ± 7.5^{b}	0.75 ± 2.1^{b}	0.76 ± 3.2^{b}
WBC (10^{3} ml^{-1})	3.6 ± 0.71^{b}	5.40 ± 1.1^{c}	4.0 ±2.1^{b}	3.3^{a}± 4.9	4.9 ± 2.7^{a}	4.7 ± 1.4^{a}	4.5 ± 6.2^{a}	4.0 ± 2.2^{b}
MCV (fl)	432.57 ± 0.7^{b}	2.1^{c} ±417	434.5 ±3^{d}	436 ± 1.1^{d}	420.9 ± 3.1^{c}	426 ± 2.2^{b}	426.1 ± 4.6^{b}	427 ± 2.8^{b}
MCH (pg)	87 ± 0.3^{b}	2.1^{a} ± 72	90 ± 2.1^{b}	91 ± 4.3^{b}	80.5 ±6.2^{b}	81.5 ± 2.3^{b}	83.8 ± 1.1^{b}	84.0 ± 3.2^{b}
MCHC (%)	19 ± 0.1^{b}	1.2^{a}± 12.0	20.2 ± 1.2^{b}	22.9 ± 4.2^{b}	18.1 ± 2.3^{b}	17.1 ± 3.2^{b}	17.9 ± 1.3^{b}	18.7 ± 3.9^{b}
Lymp (%)	65 ± 0.6^{b}	2.3^{a}± 54.1	66.6 ± 1.3^{b}	77 ± 3.2^{c}	58.1 ± 3.4^{a}	59.0 ± 5.4^{a}	61 ± 2.2^{b}	63.1 ± 2.3^{b}
Mon (%)	2.66 ± 0.5^{b}	0.1^{a} ± 3.7	2.4 ±4.2^{b}	2.2 ± 2.4^{b}	3.5 ± 1.1^{a}	3.4 ± 7.1^{a}	3.4 ± 6.3^{a}	2.9 ± 4.1^{b}
Neut (%)	27.66 ± 2.51^{b}	0.4^{a} ±39.8	29.2 ± 2.4^{b}	29.8 ± 3.3^{b}	36.6 ± 2.1^{a}	31.9 ± 1.2^{b}	31.8 ± 5.4^{b}	29.9 ± 1.5^{b}
Eos (%)	1.66±0.57^{b}	1.2^{a} ± 2.8	1.62 ±1.2^{b}	1.61 ± 4.4^{b}	2.1 ± 1.4^{b}	2.0 ± 5.2^{b}	2.0 ± 6.2^{b}	1.9 ± 4.1^{b}

Data are expressed as mean±SD. Neut: neutrophil; Mon: Monocyte; Lymp: Lymphocyte; Eos: Eosinophil. Differences in Latin letters in each row shows a significant difference (p<0.05).

Table 3: The Immunological parameters of *Common carp* fed with different levels of Garlic extract for two months then exposed with SVCV for 4-weeks (mean±SD).

Parameters	A	B	C	D	E	F	G	H
Lysozyme Activity (u ml^{-1})	39.3 ± 0.51^{b}	50.9 ± 0.2^{a}	47.9 ± 1.2^{a}	51.9 ±0.7^{a}	52.5 ± 0.5^{a}	53.3 ± 0.3^{a}	54.7 ± 0.3^{a}	55.9 ± 0.1^{a}
IgM (u ml^{-1})	29 ± 0.8^{b}	40.1 ± 0.5^{a}	37.1± 0.8^{a}	41.8 ±0.9^{a}	41.5 ± 0.2^{a}	42.2 ± 0.2^{a}	44.0 ± 0.1^{a}	45.9 ± 0.3^{a}

Differences in Latin letters in each row shows a significant difference (p<0.05). At the end of the experiment, the virus isolation has performed on 24h EPC cell culture to detect of the SVCV (Figs. 2 and 3). The cell culture results have confirmed by RT-PCR assay.



Figure 2: Negative control in EPC cell line (×100).

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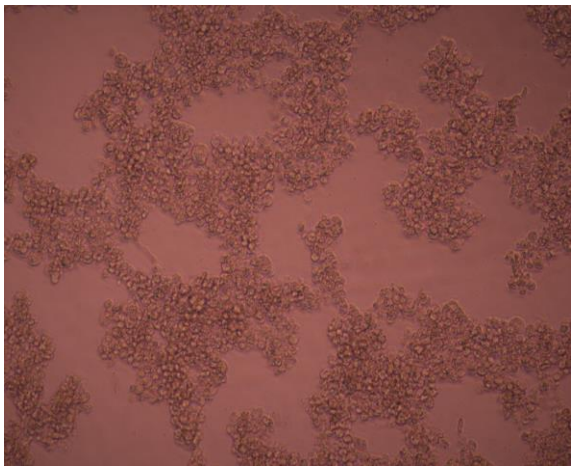


Figure 3: Cytopathic effects (CPE) of SVCV in EPC cell line (×100).

Discussion

Mortality rate

In this study, the fish fed with garlic extract (two concentrations) that have been simultaneously exposed to SVCV (groups E, G), 29% and 34% mortality have observed at the end of the fourth week respectively. Although the fish fed with garlic extract (both concentrations) that were adjacent to the SVCV (group F, H), for 4 hours had 20% and 26% mortality rate at the end of the fourth week. There was a significant difference between the two garlic-fed groups separately with group B (both concentrations) in decreasing mortality (p<0.05). In the group which have been receiving 5 g garlic extract kg^{-1} basal diet, the mortality rate was lower than the other groups (p>0.05) and the virus control group losses have reported at approximately 66% after 4 weeks. Fijan and Matsin (1980) have been reported that susceptible spring mortality are typically 40-70%, and the signs of disease and mortality may also be affected by secondary infectious and bacterial agents. In European aquaculture, losses have reported up to 70% in young carp (Ahne *et al*., 2002), but they are usually from 1 to 40% (OIE, 2018). Herbal extracts are proved to reduce mortality against pathogen challenges (Ardo *et al*., 2008). Reduction of *Aeromonas hydrophila* mortality in rainbow trout has reported by Nya and Austin (2009) fed with different dose of garlic. Similarly, adding garlic at 0.1–1 % rate in the diet of Rohu carp fingerlings for 60 days significantly reduced mortality due to Aeromoniasis (Sahu *et al*., 2007). According to Aly *et al*. (2010), an improved survival among tilapia fed on diets containing immuno stimulants or probiotics has recorded where garlic-supplemented diet resulted in a higher total harvest weight. It has been stated previously that immune stimulants improve the protection of fish against diseases by enhancing nonspecific and humoral defence mechanisms (Sakai, 1999). The reduction in fish mortality and the delay in the loss (after the use of garlic extracts in common carp fingerlings) can be attributed to the increased immune parameters of the host fish of the virus and, as a result, the resistance to pathogen.

Evaluating some blood and immune indices

Hematological changes are commonly used to determine the body status and to assess the impact of environmental, nutritional or pathological stress **(**Elagib and Ahmed, 2011; Omidvar *et al*., 2018). Strengthening the innate or non-specific immune system is very important in the fish, because they are vulnerable to many opportunistic

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bacteria and other stressors under the growing conditions, and the pathogenicity of an invading factor depends on the host's immune system's ability to fight (Dixon and Stet, 2001). The first immune response after the introduction of infectious, into the body of the fish is related to the non-specific immunity system. In fact, the increase in leukocytes is due to the power of phagocytosis and the production of antibacterial and antiviral compounds (Zorriehzahra *et al*., 2010). So far, few hemological and immunological studies have been conducted on fish viruses, but the effects of bacterial, parasitic and fungal agents on these factors is significant and comparable. In many bacterial and viral diseases including Infectious Hematopoietic Necrosis (IHN) and Erythrocytic Necrosis Virus (ENV) which leads to the severe damage to the anterior part of kidney of fish that plays a key role in the production of blood cells, a significant reduction in the number of red blood cells, hematocrit, and hemoglobin appears (Haley and Waiser, 1985). In the present study, those fish which received garlic extract (both concentrations) and exposed to SVCV (simultaneously or with 4 hours of initial adjacency of the virus and extract) as well as the group that only received SVCV (virus control), has observed that the hemoglobin, hematocrit, and RBC decreased less than the control group (without any extracts and virus). The most significant decrease has observed in the control group of the virus among the control groups (p<0.05) and the lowest reduction of the mentioned parameters has been observed in the garlic extract group at the concentration of 5 g kg^{-1} when the fish have been exposed to the virus (with 4 hours of initial adjacency of the virus and extract). Reduction in the erythrocytic count is a sign of anemia. The occurrence of anemia in SVC disease has been noted (OIE, 2018). On the other hand, the garlic extract treatment enhances the erythropoiesis as shown by the significant increase in the RBCs and Hb concentration in groups D, F, G and H as compared with the group B. This improvement in erythropoiesis may be related to the enhancement of antioxidant activity of this extract in RBCs (Al-Azzawie and Alhamdani, 2006). The group of fish that only received the virus had high levels of WBC and neutrophils (p<0.05), in the other groups which receiving garlic extract only or garlic extract prior to concurrent or 4-hour involvement with the virus, there were also an increase in WBC and neutrophils, but relatively less than a group of fish which only have been received the virus. Leukocytosis with lymphocytosis in the groups D, E, F and G may indicate an immune-stimulatory effect of this extract (Mahmoud *et al.,* 2013; Eiman *et al.,* 2014). In SVC disease, the widespread damage of hematopoietic tissues leads to anemia, leukopenia, and thrombocytopenia (Wolf, 1988) and the major changes in WBC include a significant increase in neutrophils and monocytosis (Egusa, 1992), which results in a decrease in lymphocytes. In the study of Haney *et al*. (1992) on

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Onchorhynchus keta with Erythrocytic necrosis virus diseases (VEN) and in the study of Wedemeyer *et al*. (1978) in fish with Infectious hematopoietic necrosis diseases (IHN), as same as the present study, showed an increasing in WBC with a decrease in RBC, Hb and Hct. In the study of Barham *et al*. (1980), the number of RBC, Hemoglobin, and Hematocrit has lowered in the Cham fish infected with *Vibrio anguillarum* too. Martins *et al*. (2012) has investigated the changes of the hematology of Nile tilapia and the effect of bacterial infection of Enterococcus sp. It has observed that in infected fish, the number of WBC, neutrophils and hematocrit increased significantly. In the present study, the group of fish that has been received only extract (with both concentrations) without exposure to the virus, as well as in a group of fish that has been received an eight-week extract with exposure to the SVCV, either simultaneously or adjacent with the extract four hours before exposure, showed higher levels of lysozyme (p<0.05). Lysozyme is a non-specific immune factor in vertebrates that secretes from white blood cells, especially neutrophils, monocytes, and macrophages, and indicates leukocytes activity which increases with the increased activity of phagocytosis of leukocytes (Itami *et al*., 1992; Yano, 1996; Sheikhzadeh, 2013). In this study, an increase of lysozyme activity has been shown which is in agreement with several reports indicating the role of herbal immune stimulants in enhancing lysozyme activity (Rao *et al*., 2006; Choi *et al*., 2008; Haghighi *et al*., 2014). In the present study, a group of fish that has been received only extract (with both concentrations) without exposure to the virus, as well as in a group of fish that received an eight-week extract with exposure to the SVCV, either simultaneously or adjacent with the extract four hours before exposure, showed higher levels of IgM (p<0.05). B-lymphocytes produce IgM, in response to systemic antigenic stimuli for example in viral diseases (Hansen *et al*., 2005). The highest levels of lysozyme and IgM have observed in the fish fed with extract of 5 g kg^{-1} and were exposed to the SVCV which was adjacent to the extract 4 hours before exposure (p<0.05). At the end of the experiment, the cell culture on EPC cell line was performed to determine that the fish mortalities were related to the SVC or not. The cell culture results have been confirmed by RT-PCR technique.

Based on the results, it was determined that the extract of garlic (in both concentrations, especially in the concentration of 5 g kg^{-1}) could reduce undesirable changes in WBC and RBC while increased immunity parameters, delayed and reduced mortality rate in the fish exposed to the SVCV (especially when the virus was adjacent to the extract 4 hours before exposure).

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