Effects of diets containing dry extracts of *Achillea millefolium*, *Mentha piperita* and *Echinacea purpurea* on growth, hematological and immunological indices in juvenile common carp (*Cyprinus carpio*)

S Alinezhad*

Institute of Agricultural Education and Extension, Agricultural Research Education and Extension Organization (AREEO), Tehran, Iran

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Abstract

In this study, the effects of three herbal dry extracts (*Achillea millefolium*, *Mentha piperita* and *Echinacea purpurea*) were investigated on growth, hematological and immunological indices in juvenile common carp (*Cyprinus carpio*). 400 juvenile fish with initial weight of 14.30 ± 0.77g were studied in 10 treatment groups (9 treatment groups & a control) with four replicates for 60 days. Three levels (0.1, 0.5 and 1%) of dry extracts of each herb were prepared according to standard method and added to the commercial common carp feed. At the end of period twelve fish collected out of each group and the parameters were measured. In order to the results, weight gain, specific growth rate (SGR) and complement C4 were not affected by dietary treatments (P>0.05). Red blood cell (RBC) counts in 0.5 and 1%-diet groups as well as Hemoglobin in three levels of all herbs was increased (P≤0.05). Hematocrit in 0.5%, 1%-diet *M. piperita* and *E. purpurea* groups was shown significant increases (P≤0.05). Mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) in all groups except 0.1% *M. piperita* group and 0.5% *E. purpurea* were increased compare with control group (P≤0.05). MCHC in 0.5% *E. purpurea* and 0.1 and 1% *M. piperita* groups showed the highest values. Levels of 0.5% *M. piperita* and 1% *E. purpurea* and *A. millefolium* make significantly increases in total leukocytes and neutrophils (P≤0.05). Significantly increases of lymphocytes and decrease of monocytes were observed in levels of 0.5% *E. purpurea* and 1% level of all herbs groups (P≤0.05). Increased levels of immunoglobulin compared to control were significant only in 1% level of all herbs (P≤0.05). Complement C3 was also increased 1% of *A. millefolium* and *M. piperita* groups compared to the control (P≤0.05). All levels of *A. millefolium* and *M. piperita* and 1% *E. purpurea* groups caused a significant increase in lysozyme concentration compare with the control (P≤0.05). The results indicated all three herb extracts in diet can improve immune responses and hematological parameters in common carp. Comparing these extracts, the *M. piperita* extract with a lower concentration is more efficient.

***Correspondence** S Alinezhad, Institute of Agricultural Education and Extension, Agricultural Research Education and Extension Organization (AREEO), Tehran, Iran (e-mail: soheilalinezhad47@gmail.com).

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Introduction

The immunostimulants include a group of biological and synthetic compounds that enhance the innate immunity in animals and protect them from pathogens (Bairwa, Jakhar, Satyanarayana & Reddy 2012). Regarding the mechanism of action on immune system, these stimulants have more effective response in lower vertebrates rather than higher vertebrates.

On the other hand, the tendency to eliminate antibiotics in aquaculture due to high costs, drug resistance, environmental and administrative problems has led to more attention to safe stimulants as an alternative method (Harikrishnan, Kim, Kim, Balasundaram & Heo 2011). The use of herbal immune-stimulant compounds has multiple advantages, including the lesser side effects of organisms and environment, lack of drug resistance, low price, sustained and affordability and attracted a lot of attention globally, especially in developed countries (Vaseeharan & Thaya 2014). Common carp (*Cyprinus carpio*) is a bony fish and one of the most popular fish in many countries. This fish is widely cultured in Europe and Asia and also Iran (Toral-Granda, Lovatelli & Vasconcellos 2008, Iran Fisheries Organization 2017).

In several studies, the positive effects of medicinal plants were studied on common carp growth and immunity improvements. In such researches, weight gain amelioration, FCR decrease, specific growth rate (SGR), leukocytosis, lysozyme activity enhancement, increase of phagocytic activity and plasma globulin value after usage of herbs extracts in carp species feed were reported. Mixture of *Ocium bacilicum, Mentha piperita, juglans regia* and *Cinnamomum zeylanicum* extracts (Hajibeglou & Sudagar 2010), Mixture of *Astragalus membranaceus, Polygonum multiflorum, Isatis tinctoria* and *Glycyrrhiza glabra* extracts (Yuan, Chen, Sun, Wu, Gong & Han 2007), *Zataria multifloria* (Soltani, Sheikhzadeh, Ebrahimzadeh-Mousavi & Zargar 2010), *Olea eurcpaea* (Karimi pashaki, Ghasemi, Zorrieh Zahra, Shrif Rohani & Hosseini 2018) had positive effects on growth, hematological and immunological indices in common carp.

Achillea millefolium, *Mentha piperita* and *Echinacea purpurea* are perennial plants that have antioxidant, anti-bacterial, anti-inflammatory and immunostimulant activities (Hajibeglou & Sudagar 2010, Sharif Rohani, Pourgholam & Haghighi 2016, Aly & Mohamed 2010, Candan, Unlu, Tepe, Daferera, Polissiou, Sökmen & Akpulat 2003).

In this research, in order to the importance of this medicinal herbs in immunity and fish production, the effects of different concentrations in *A. millefolium*, *M. piperita* and *E. purpurea* extracts in diet were studied on the growth, hematological and immunological indices in common carp juveniles.

Materials and methods

Extraction method

The studied plants were got from the Iranian Institute of Medicinal Plants. After drying the plants in shade and mild air, the samples were

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powdered. In each extraction time, 100 g of powder was poured in a laboratory balloon containing the ratio of 1:1 distilled water and 99.6% ethanol (300 ml of each substance) and was gently mixed in a shaker for 48 hours. The mixture was then filtered with a Whatman 42 filter paper and a Buchner funnel. After finishing extraction, the extracts obtained by rotary device were concentrated at 40-50°C. The concentrated extract was dried in oven at 40°C for two days. Then, they were kept in autoclaved glass containers at 3ºC until they were mixed with the commercial diet (Sivam 2001).

Preparation of treatments

Three levels (0.1, 0.5 and 1%) of dry extracts of each medicinal herb were added to the commercial common carp feed (Faradaneh, Aquatic Animal Feed Producer) (table 1). In order to add dried extract to the diet, the total feed intake at each group was estimated, and then the dry extract was added to the diet with a specified volume of distilled water (40 ml) to obtain a paste product. It was finally dried at room temperature at 30°C for 48h. All diet groups were stored at -20°C until used (Akbary, Kakoolaki, Salehi, Zorriehzahra, Sepahdari, Mehrabi 2016). Totally ten groups with four replicates were prepared (one commercial diet group without additives as control group).

Table1. Approximate analysis (%) of juvenile common carp feed

Chemical analysi	juvenile feed
crude protein	35-38
fat	4-8
Fiber	3-6
Ash	7-11
Moisture	5-11
Phosphorus	1-1.5
Size of floating pellet (mm)	2

General Culture Conditions

The number of 400 juvenile common carp with a weight of 14.30 ± 0.77 g were separated from the original stock and kept in ten groups in forty 200-litered aquaria.

The daily water change rate was 10% for each aquarium. The water temperature was 26-28°C, dissolved oxygen was 5.08-7.70 ppm, pH was 7.9 ± 0.3 and ammonia was less than 0.2 ppm. After a week of adaptation with commercial common carp diet (without additives), the treatment diets were given to fish for 60 days. Fish were fed satiated by experimental diet. Feeding times were eight times per day in the first three weeks and 4 times per day from fourth week until the end of the period. At the end of period, three fish were taken from each replicate for the tests (e.g. 12 fish from each treatment).

Sample collection and analysis

Feeding was stopped one day before blood sampling. At the end of period, three fish of each replicate were randomly caught to measure the blood parameters. They were anesthetized by 100 ppm clove extract (Javaheri, Nekoubin & Haji Moradlu 2012). Blood sampling was done from caudal vein that were stored in EDTA-containing tubes at 4°C until testing (Blaxhall & Daisley 1973). Measured growth indices included initial, final weights, weight gain and specific growth rate (SGR), which is calculated based on the following formula (Watanabe, Ernest & Chassar 1993).

$$SGR\% = \left({}^{L_n W_1 - L_n W_0}/_{\text{Experimental period (Day)}}\right) * 100$$

L_nW_1: Natural log of final weights, L_nW_0: Natural log of final weights

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Hematological parameters including hematocrit (Rehulka 2000), red blood cell counts (RBC), hemoglobin value (Blaxhall and Daisley 1973), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) (Klinger, Blazer & Echevarria 1996) were determined.

The measured immune parameters consist of white blood cell count (WBC) by Neubauer chamber method (Thrall, Weiser, Allison & Campbell 2012), separation detection of a variety of white cells by the extension method with Wright-Giemsa stain (Sharif-Rohani *et al.* 2016), measuring immunoglobulin and C3 and C4 enzymes complement system by commercial kits (Authorized by: Eurolyser, Belgium) Pars Azmoon Co. (Shahsavani, Mohri & Gholipour Kanani 2010) and the rate of lysozyme activity by turbidity method in ELISA (Giri, Sen & Sukumaran 2012).

Statistical Analysis

The design was carried out in ten treatments and four replicates as a completely randomized design with three observations per replication.

Finally, the design was analyzed with SPSS (version 21, Chicago, USA) software by One-Way ANOVA method to evaluate the effects of treatments at 0.05. The Kolmogorov-Smirnov (K-S) test for the normality of data distribution and the Duncan's multiple range test comparison of the means were used at 0.05.

Results

Growth indices in juvenile common carp

In order to the juvenile common carp biometry results (table. 2) no measured parameters showed significant differences among treatments (P>0.05).

Table 2. Growth indices of juvenile common carp fed with different levels of *Achillea millefolium* (A.m), *Mentha piperita* (M.p) and *Echinacea purpurea* (E.p) after 60 days (Mean ± SD)

Treatment	initial weight (g)	final weight (g)	weight gain (g)	specific growth rate (%)
Control	14.07 ± 0.81	78.08 ± 2.88 [a]	63.45 ± 2.85 [a]	2.66 ± 0.11 [a]
0.1% A. m	13.80 ± 1.02	76.90 ± 3.01 [a]	64.05 ± 4.12 [a]	2.77 ± 0.11 [a]
0.5% A. m	14.87 ± 0.74	81.09 ± 1.85 [a]	66.66 ± 2.01 [a]	2.33 ± 0.14
1% A. m	15.02 ± 0.85	80.77 ± 2.94 [a]	64.32 ± 3.33 [a]	2.65 ± 0.12 [a]
0.1% M. p	14.76 ± 0.66	77.85 ± 3.88 [a]	62.85 ± 3.75 [a]	2.45 ± 0.10 [a]
0.5% M. p	13.90 ± 1.33	76.05 ± 2.66 [a]	62.66 ± 3.01 [a]	2.72 ±0.09 [a]
1% M. p	14.06 ± 0.94	75.45 ± 2.77 [a]	62.12 ± 2.88 [a]	2.46 ±0.11 [a]
0.1% E. p	14.45 ± 0.64	77.56 ± 1.85 [a]	66.81 ± 2.24 [a]	2.85 ±0.09 [a]
0.5% E. p	14.82 ± 1.08	82.33 ± 1.25 [a]	65.90 ± 1.89 [a]	2.77 ±0.12 [a]
1% E. p	14.95 ± 0.72	81.66 ± 2.55 [a]	63.45 ± 2.89 [a]	2.55 ±0.13 [a]

Uncommon letters in columns indicate a significant difference at the level of 0.05.

Hematological indices

The results of blood indices are shown in table3 and 4.

Results and changes of RBC, hemoglobin (Hb) and hematocrit are shown in table 3. In order to the results, there was a significant difference among 0.5 and 1% of three herbs levels and control groups for RBC count (P≤0.05). But there was no significant difference between 0.1% and control group in

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this index. Hb amount in all treatments were significantly higher than the control (P≤0.05). 0.5 And 1% levels of *M. piperita* and *E. purpurea* caused significant increase in hematocrit (P≤0.05).

Table 3. RBC, Hemoglobin (Hb) and Hematocrit of juvenile common carp fed with different levels of *Achillea millefolium* (A.m), *Mentha piperita* (M.p) and *Echinacea purpurea* (E.p) after 60 days (Mean ± SD)

Treatment	RBC ($\times 10^6$ mm^{-3})	Hb (g dl^{-1})	Hematocrit (%)
Control	1.29 ± 0.04 ^{a}	6.95 ± 0.07 ^{a}	34.22 ± 0.67 ^{a}
0.1% A. m	1.31 ± 0.09 ^{a}	7.25 ± 0.22 ^{b}	36.02 ± 0.42 ^{ab}
0.5% A. m	1.40 ± 0.06 ^{b}	8.10 ± 0.20 ^{c}	38.28 ± 0.50 ^{b}
1% A. m	1.42 ± 0.01 ^{bc}	8.02 ± 0.35 ^{c}	39.4 ± 0.24 ^{b}
0.1% M. p	1.34 ± 0.08 ^{ab}	7.35 ± 0.15 ^{b}	33.51 ± 0.56 ^{a}
0.5% M. p	1.43 ± 0.04 ^{bc}	8.15 ± 0.16 ^{c}	39.68 ± 0.35 ^{b}
1% M. p	1.45 ± 0.09 ^{c}	7.90 ± 0.08 ^{bc}	40.61 ± 0.54 ^{b}
0.1% E. p	1.33 ± 0.04 ^{ab}	7.50 ± 0.21 ^{c}	36.48 ± 0.77 ^{ab}
0.5% E. p	1.43 ± 0.05 ^{bc}	7.65 ± 0.10 ^{c}	40.55 ± 0.46 ^{b}
1% E. p	1.41 ± 0.01 ^{b}	8.00 ± 0.08 ^{bc}	39.31 ± 0.36 ^{b}

Uncommon letters in columns indicate a significant difference at the level of 0.05.

Changes in MCV, MCH and MCHC through treatments are shown in table 4.

In MCV, there was significant increase among all treatments and the control, except 0.1% *M. piperita* level (P≤0.05) that showed the lowest amount. MCH, in all *A. millefolium* levels, 0.5 and 1% *Mentha piperita* and 0.1 and 1% *E. purpurea* levels was significantly higher than the control (P≤0.05). MCHC results were contradicted and 0.5 *E. purpurea* and 0.1 and 1% *M. piperita* levels showed the highest MCHC (P≤0.05).

Table 4. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) of juvenile common carp fed with different levels of *Achillea millefolium* (A.m), *Mentha piperita* (M.p) and *Echinacea purpurea* (E.p) after 60 days (Mean ± SD)

Treatment	MCV (Fl)	MCH (Pg)	MCHC (%)
Control	266.27 ± 1.92 ^{a}	53.87 ± 0.50 ^{a}	20.30 ± 0.06 ^{b}
0.1% A. m	274.45 ± 2.50 ^{b}	55.34 ± 0.42 ^{b}	20.12 ± 0.10 ^{ab}
0.5% A. m	273.64 ± 1.89 ^{b}	57.85 ± 0.65 ^{b}	21.15 ± 0.07 ^{b}
1% A. m	277.36 ± 2.56 ^{b}	56.47 ± 0.71 ^{b}	20.35 ± 0.07 ^{b}
0.1% M. p	250.37 ± 3.84 ^{c}	54.85 ± 0.65 ^{a}	21.93 ± 0.08 ^{c}
0.5% M. p	278.42 ± 1.43 ^{b}	56.99 ± 0.55 ^{b}	20.53 ± 0.05 ^{b}
1% M. p	280.12 ± 1.73 ^{c}	54.48 ± 0.48 ^{b}	19.45 ± 0.07 ^{a}
0.1% E. p	274.18 ± 2.76 ^{b}	56.40 ± 0.78 ^{b}	20.55 ± 0.05 ^{b}
0.5% E. p	283.53 ± 1.77 ^{c}	53.49 ± 0.55 ^{a}	19.86 ± 0.04 ^{a}
1% E. p	278.67 ± 1.83 ^{bc}	56.73 ± 0.49 ^{b}	20.35 ± 0.08 ^{b}

Uncommon letters in columns indicate a significant difference at the level of 0.05.

Immunological indices

The results of the WBC and ratio of leukocytes in the samples show in table 5. The results showed that there was no significant difference in the WBC and ratio of leukocytes between the levels of 0.1% of all three plants and the control group. However, there was a significant difference between the higher levels of treatments and the control in some parameters. Levels of 0.5% *M. piperita* and 1% *A. millefolium* and *E. purpurea* caused a significant increase (P≤0.05) in total numbers

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of leukocytes and neutrophils percent. Levels of 0.5% *E. purpurea* and 1% of all three plants significantly increased lymphocytes and significantly decreased monocytes (P≤0.05). The percentage of eosinophil was not affected by treatments.

Table 5. Total WBC and percentage of WBCs of juvenile common carp fed with different levels of *Achillea millefolium* (A.m), *Mentha piperita* (M.p) and *Echinacea purpurea* (E.p) after 60 days (Mean ± SD)

Treatment	Eosin.%	Mono. %	Neutr. %	Lymph. %	WBC. mm^{-3}
Control	0.40 ± 0.035^{a}	3.25±1.00^{b}	4.73±0.45^{a}	90.65±1.53^{a}	43883.69±2598.60^{a}
0.1% A. m	0.55± 0.048^{a}	3.03±0.55^{b}	4.53±0.53^{a}	91.45±1.73^{ab}	42256.00±2676.68^{a}
0.5% A. m	0.39± 0.082^{a}	3.18±0.36^{b}	5.23±0.81^{ab}	87.96±2.04^{a}	45794.67±2292.79^{a}
1% A. m	0.50±0.075^{a}	2.16±0.83^{a}	5.85±0.81^{b}	93.48±1.52^{b}	57809.15±2845.85^{b}
0.1% M. p	0.44±0.101^{a}	2.96±0.93^{b}	4.73±1.00a	88.30±3.78^{a}	42775.44±2787.29^{a}
0.5% M. p	0.38±0.076^{a}	2.82±0.93^{ab}	5.73±0.51^{b}	92.05±1.08^{b}	54766.00±3637.64^{b}
1% M. p	0.48±0.101^{a}	1.41±0.11^{a}	5.20±0.41^{ab}	94.75±2.16^{b}	51565.00±1527.03^{ab}
0.1% E. p	0.51±0.064^{a}	3.25±0.64^{b}	4.86±0.58^{a}	92.58±1.58^{ab}	42180.94±3288.29^{a}
0.5% E. p	0.47±0.046^{a}	2.17±1.25^{a}	4.65±0.99^{a}	93.55±1.85^{b}	50009.86±5663.66^{ab}
1% E. p	0.40±0.091^{a}	1.73±0.91^{a}	5.58±0.89^{b}	93.82±1.12^{b}	58891.52±5098.60^{b}

Uncommon letters in columns indicate a significant difference at the level of 0.05.

Table 6 shows the mean and standard deviation of Total immunoglobulin, C3 and C4 and lysozyme. Increasing the levels of immunoglobulins were significant only in high level groups (groups contain 1% extract in all three plants (P≤0.05) compared to control and in other levels, there was no significant difference. Among the treatments no significant difference for immunoglobulins levels were observed.

Complement system enzymes were also partially affected by treatments, so that C3 at 1% levels of *A. millefolium* and *M. piperita* had a significant increase compared to the control (P≤0.05). In C4, no significant difference was observed between the treatments.

Differences in the lysozyme activity were significant. The lysozyme activity was increased significantly in all levels of *A. millefolium* and *M. piperita*, as well as 0.5% and 1% of *E. purpurea* levels, compared to the control (P≤0.05). The results of comparison between the treatments also showed that triple levels of *M. piperita* and 1% of *E. purpurea* caused a significant increase in the amount of lysozyme compared to the triple levels of *A. millefolium* and 0.1% of *E. purpurea* (P≤0.05).

Table 6. Immune system proteins in common carp blood serum fed with different levels of *Achillea millefolium* (A.m), *Mentha piperita* (M.p) and *Echinacea purpurea* (E.p) after 60 days (Mean ± SD)

Treatment	LYS (µg ml^{-1} min^{-1})	C_4 (mg dl^{-1})	C_3 (mg dl^{-1})	Ig (mg ml^{-1})
Control	5.16 ± 0.44^{a}	5.35 ± 1.99^{a}	35.08 ± 6.52^{a}	15.60 ± 1.34^{ab}
0.1 % A. m	6.87 ± 0.34^{b}	4.85 ± 2.05^{a}	35.53 ± 5.44^{a}	14.65 ± 2.68^{a}
0.5% A. m	6.58 ± 0.56^{b}	6.04 ± 1.55^{a}	39.21 ± 5.23^{a}	15.35 ± 1.59^{ab}
1% A. m	6.58 ± 0.67^{b}	6.55 ± 0.91^{a}	46.33 ± 3.85^{b}	16.39 ± 2.08^{b}
0.1% M. p	7.62 ± 0.65^{c}	6.44 ± 2.43^{a}	27.50 ± 4.63^{a}	14.02 ± 2.39^{a}
0.5% M. p	8.14 ± 0.85^{c}	8.33 ± 3.11^{a}	38.43 ± 5.38^{a}	16.60 ± 1.49^{b}
1% M. p	7.91 ± 0.33^{c}	7.80 ± 2.05^{a}	46.57 ± 7.21^{b}	17.36 ± 0.99^{b}
0.1% E. p	6.37 ± 0.45^{ab}	7.65 ± 1.95^{a}	33.33 ± 9.64^{a}	14.94 ± 1.95^{a}
0.5% E. p	7.44 ± 0.25^{bc}	6.04 ± 3.02^{a}	37.24 ± 3.09^{a}	13.96 ± 1.92^{a}
1% E. p	8.56 ± 0.31^{c}	7.56 ± 2.46^{a}	39.08 ± 7.11^{a}	14.64 ± 2.29^{b}

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Discussion

The results showed that all three herbal extracts have significant efficacy on many immunological and hematological parameters in juvenile common carp in higher concentration.

Growth indices

According to the present results, growth indices did not show significant change. Several researches about medicinal plants on growth indices showed different consequences on growth indices. In some papers, medicinal herbs affect positively on growth indices in juvenile common carp, for example, effective ingredient of *Macleaya cordata* could cause weight gain and increase of SGR (Imanpoor, Salaghi, Roohi, Beikzadeh & Davoodipoor, 2015), Alovera (Alishahi, Ranjbar, Ghorbanpour, Peyghan, Mesbah & Razi jalali 2010) and aqueous-alcoholic extract of olive leaf (Karimi Pashaki *et al.* 2018) have such consequences on growth indices. Adding *E. purpurea* extract to diet of rainbow trout, *Oncorhynchus mykiss* (Oskoii, Kohyani, Parseh, Salati, and Sadeghi 2012) and angel fish, *Pterophyllum scalare* (Kasiri, Farahi & Sudagar 2011) caused growth indices improvement.

M. piperita extract was also improved growth indices in Caspian kutum, *Rutilus frisii kutum* (Adel, Safari, Monji, Farabi 2015.a). Extracts mixture of four medicinal plants contained *M. piperita* in common carp juvenile had similar results (Hajibeglou & Sudagar 2010). Barreto, Menten, Racanicci, Pereira. and Rizzo (2008) claim that growth promotion effect of herbal extracts depends on appropriate concentration, ration ingredients, the plant's alkaloid and farming management. For instance, the use of garlic extract does not show significant increase on tilapia growth characteristics for two months, but period protracting to eight months showed significant increase (Aly, Atti & Mohamed 2008). In order to our results, in 0.5% of all herb concentrations, weight gain and growth rate was higher than the others.

Hematological indices

In order to our results, RBC count in 0.5 and 1% levels and hemoglobin in all herbs levels were significantly higher than the control. Hematocrit in 0.5 and 1% *M. piperita* and *E. purpurea* have significant increases. MCV in all herbs levels (except 0.1% of *M. piperita*), MCH in all levels of *A. millefolium* and 0.5 and 1% *M. piperita* levels as well as 0.1 and1% *E. purpurea* were significantly higher than the control. In spite of two latter indices, results for MCHC were different, and 0.5% *Echinacea purpurea* and 0.1 and 1% *Mentha piperita* showed the highest values. Blood is one of the most sensitive and vital tissue in live animals, that many tissues responses to biological and environmental factors can be seen in blood. Nowadays, in medical hematology, hematological indices are the major paraclinical tools for infectious and noninfectious diagnosis (Ahmadifar, Akrami, Ghelichi & Mohammadi Zarejabad 2011). There are different consequences of diet-usage herbal extracts on cyprinid fishes hematological indices. In Karimi Pashaki *et al.* (2018) research aqueous-

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alcoholic extract of olive leaf has no significant on RBC, Hb, hematocrit, MCV, MCH and MCHC indices in juvenile common carp. Goldfish fed *Urtica dioica* extract did not show significant differences on same indices, too (Nejad Moghaddam, Imanpoor, Jafari & Safari 2018). In another study, *Macleaya cordata* extract could not maintain hematocrit value against salinity in common carp (Imanpoor *et al.* 2015). Studies on *E. purpurea* and *M. piperita* extracts in *Mugil cephalus* feed showed significant increases in RBC, hematocrit and hemoglobin (Akbary *et al.* 2016). In Alishahi, Mesbah, Namjouyan, Sabzvaryzadeh & Razi-Jalali (2012) study, *Echinacea purpurea* extract significantly increased hematocrit and hemoglobin in *Aeromonas hydrophila*-infected Oscar fish. In one research on Nile tilapia, *Pseudomonas flourscens*-injected treatment had higher hematocrit compare to the control (Gabor, Sara & Barbu 2010). In Adel, Safari, Pourgholam, Zorriehzahra & Esteban (2015.b) study *Mentha piperita* extract made significant increase in Caspian brown trout RBC count, hematocrit and hemoglobin. Generally, hematological indices changes depends on species, age, genus, seasonal changes, geographic region, nutrition, environmental stressors, pollutions and fish reproduction cycle (Mercaldo-Allen, Dawson, Kuropat & Kapareiko 2003). Efficacy of medicinal herbs on immunological and hematological indices is consistent with plant incidence, effective ingredient and its alkaloid. Some herbal species such as used in present research, can improve nonspecific immunity and hematologic indices, because of ingredients like as Alkids and Caffeic Acid derivatives (Akbary *et al.* 2016). Increasing in RBCs, hematocrit and Hb is vital for increment of oxygen resources in biological and environmental stresses (Ruane, Wendelaar Bonga & Balm 1999). Increase of RBC and consequently smaller size are indicators of decreasing oxygen diffusion into the tissues. In other words, by enhancement of RBC amount, oxygen entering into gills and tissues oxygenation will increase (Hosseini, Oraj, Yegane & Shahabi 2014). In control group, less Hb amount and less oxygen take up consequently may cause lower blood oxygen levels and limitation for their tissues oxygenation in stress conditions.

Immunological indices

Statistical analysis showed that the changes in lymphocytes, monocytes and neutrophils are significant. 0.5% *M. piperita* and 1% *A. millefolium* and *E. purpurea* levels in diet, significantly increased total WBCs and neutrophils percentage. 0.5% *E. purpurea* and 1% of all extracts levels significantly increased lymphocytes and decreased monocytes values. The results are consonant with previous researches and indicated immunity improvement by prescribing such herbal extracts.

Such a situation is also reported following the administration of *M. piperita* powder in the diet of sea bass fish, *Lates calcarifer* (Talpur 2014) and Caspian brown trout, *Salmo trutta caspius* (Adel *et al.* 2015.b). Oral consumption of *E. purpurea* in Tilapia fish (*Oreochromis niloticus*) also caused a significant increase in the number of leukocytes (Aly & Mohamed 2010).

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In another study, 0.1% of *E. purpurea* extract had no significant impact on WBC and their ratio (Sharif-Rouhani *et al.* 2016). The effect of mixed extracts of four herbs contained *M. piperita* in the diet of common carp caused effective improvement of nonspecific immunity and WBCs (Hajibeglou & Sudagar 2010). However, the use of Aloe Vera extract in common carp diet had no significant impact on cellular immunity (Alishahi *et al.* 2010). In two separate studies, the extracts of henna (Soltanian & Fereidouni 2016) and spurge leaf (Pratheepa & Sukumaran 2014) were used in common carp to improve immune system. In studies, WBC value, number of lymphocytes, monocytes and neutrophils were increased significantly. WBCs are the first barrier against pathogens and their quantities and proportion show their functions in immune system. Researches indicated, administration of herbal extracts in feed can cause viability of fish against pathogens (Nya & Austin 2011). Regarding the results of present and former researches, the most probable reason of such changes are the herbs effects on immunity and control of bacterial diseases either prior or after encountering disease (Thrall *et al.* 2012). The rise in WBCs following the use of plant stimulants seems to be dependent on β-glucans that can detect specific receptors on WBCs. When these receptors are occupied by glucans, the activity of T cells and monocytes in enveloping, killing and digestion of pathogenic bacteria becomes greater, all of which improve the host defense system (Andrews, Sahu, Pal & Kumar 2009).

Regarding the present results, there is significant increase in immunoglobulin levels in 1% of three herbs treatments, C3 in 1% of *A millefolium* and *M piperita*, lysozyme activity in all levels of *A. millefolium* and *M. piperita* as well as 0.5 and 1% of *E. purpurea*. Immunoglobulins levels were significantly higher in groups contained 1% of three herbs extracts compare with the control. C4 level variation was like as C3 changes, but not significant.

The pattern of complement enzymes modifications largely followed the changes in immunoglobulin; this was predictable due to the dependence of the complement system on immunoglobulins (Nonaka & Smith 2000). Several experiments have been carried out on effects of various herbs on immune system proteins in aquatic species that are consistent with the results of this test. For example, the extract of *Echinacea purpurea* stimulated the non-specific immune factors of common carp (Alishahi, Soltani, Mesbah & Rad 2011). The increase of serum lysozyme activity has also been reported in common carp (Jian & Wu 2004) after oral administration of natural immune stimulants, vaccines and some probiotics. Milk thistle seed powder has shown such effect on common carp (Harikrishnan, Nisha Rani & Balasundaram 2003). However, the use of dietary *Spirulina platensis* had no increase in levels of immunoglobulin and lysozymes of carp (Watanuki, Ota, Tassakka Kato & Sakai 2006). Concentrations of 30-120 micrograms per liter of eucalyptus oil had no effect on immunological parameters of common carp (including antibody titeration and lysozyme activity). This is probably due to the low water temperature in breeding system

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(Sheikhzadeh, Soltani, Ebrahimzadeh-Mousavi, Shahbazian & Norouzi 2011). In another study, 0.1% of *Echinacea purpurea* caused a significant increase in C3 and lysozyme in rainbow trout, but did not affect on immunoglobulins and C4 levels (Sharif-Rouhani *et al.* 2016).

In two separate studies, Henna extracts (Soltanian & Fereidouni 2016) and spurges leaf (Pratheepa & Sukumaran 2014) was used to improve the immunity of common carp. In both studies, lysozyme and immunoglobulins levels were also affected by the treatment.

Active ingredients as herbal essential oils have played an effective role in improving of immune system. Most of the results in previous studies are consistent with the results of the present study and indicate an improvement in common carp cellular immunity.

The major part of the identified compounds in the *Achillea millefolium* plant is monoterpenes. Camphor, alpha-pinene, linalool and cineol are the most important compounds known in *Achillea millefolium* essential oil, which camphor is the main essential oil (Ardestani & Yazdanparast 2007).

In a study, the most important compounds of the main essence that have antioxidant and antimicrobial properties are cineol, camphor and borneol, which is highly present in *Achillea millefolium* essential oil (Radulović, Dekić, Ranđelović, Stojanović, Zarubica & Stojanović-Radić 2012).

Previous studies have shown that many herbal essential oils of mint plants contain antioxidants and immunostimulants. The main volatile compounds identified in the mint essential oil include menthol, thymol, mentofuran, methyl acetate, cineol and menthon. Among them, thymol, menthol and paracymon are the most important components with antimicrobial activity and immunostimulation in *Mentha piperita* essential oil. The most important properties of cineol and menthol are antimicrobial and antioxidant properties. Linalool and eugenol in *Mentha piperita* essential oil have antioxidant and antimicrobial activity (McKay & Blumberg 2006).

The active ingredients in *E. purpurea* include phenolic compounds, cichoric acid, hetero polysaccharides, and alkyl amides. All three categories of these materials have increased the activity of macrophages in animal tissues (Luettig, Steinmüller, Gifford, Wagner & Lohmann-Matthes 1989). Increased levels of interleukin-2 and total number of leukocytes were observed in rat due to the extract of this plant (Goel, Chang, Slama, Barton, Bauer, Gahler & Basu 2002). The polysaccharide arabinogalactan in the *Echinacea purpurea* essential oil is known as an activator of the macrophage system in exogenous conditions (Cundell, Matrone, Ratajczak & Pierce 2003). In addition, the production of tumor necrosis alpha, interleukin-1, and interferon beta-2 and oxygen free radicals was increased by macrophages (Luettig *et al*. 1989). Cichoric acid is a phenolic compound derived from caffeic acid, which according to studies has antioxidant properties. It also stimulates the cellular immunity and consequently, non-specific immunity (El-Refaei & El-Naa 2011).

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Conclusion

According to the results, growth, hematological and immunological indices improved by feeds containing these three plants. The results of this study showed that the dry extracts of all three herbs of *Achillea millefolium*, *Mentha piperita* and *Echinacea purpurea* could improve hematological parameters and immune responses in common carp. However, *Mentha piperita* has higher efficacy with lower concentration. The presence of various phenolic, terpenoid and other compounds in the essential oil of medicinal plants can partly explain the increase of immune parameters of common carp in this study. Finally, we need more basic and farm studies to do, to provide the final recommendation for commercial use of these plants in the common carp diet.

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تأثیر عصاره خشک سه گیاه دارویی بومادران، نعناع فلفلی و سرخارگل بر شاخص‌های رشد، فراسنجه‌های خونی و شاخص‌های ایمنی ماهی کپور معمولی (*Cyprinus caprino*)

سهیل علی نژاد*

مؤسسه آموزش و ترویج کشاورزی، سازمان تحقیقات، آموزش و ترویج کشاورزی، تهران، ایران

چکیده

در این مطالعه، اثرات عصاره خشک سه گیاه دارویی بومادران (*Achillea millefolium*)، نعناع فلفلی (*Mentha piperita*) و سرخارگل (*Echinacea purpurea*) بر شاخص‌های خونی، ایمنی غیر اختصاصی و شاخص‌های رشد بچه ماهی کپور معمولی بررسی شد. ۴۰۰ بچه ماهی با میانگین وزن اولیه ۰/۷۷±۱۴/۳۰ گرم به مدت ۶۰ روز در ۱۰ تیمار (۹ تیمار حاوی افزودنی و یک تیمار شاهد) قرار گرفتند و هر تیمار دارای چهارتکرار بود. سه سطح (۰/۱، ۰/۵ و ۱ درصد) عصاره‌ی خشک گیاهان به روش استاندارد تهیه شده و به جیره تجاری ماهی کپور اضافه گردید. در پایان از هر تیمار ۱۲ قطعه برداشت شده و شاخص‌های رشد ، خون‌شناسی و ایمنی اندازه‌گیری شد. براساس نتایج، شاخص‌های افزایش وزن بدن، شاخص رشد ویژه (SGR) و C_4 سیستم کامپلیمنت تحت تأثیر تیمارها قرار نگرفتند ($P>0.05$). میزان گلبول قرمز (RBC)، در سطوح ۰/۵٪ و ۱٪ و میزان هموگلوبین، در تمامی سطوح سه تیمار، بالاتر از تیمار شاهد بود ($P\leq0.05$). هماتوکریت در سطوح ۰/۵٪ و ۱٪ نعناع فلفلی و سرخارگل افزایش نشان داد ($P\leq0.05$). میزان MCV و MCH در تمامی تیمارها، به جز سطح ۰/۱٪ عصاره نعناع فلفلی و ۰/۵٪ سرخارگل بالاتر از تیمار شاهد بودند ($P\leq0.05$). MCHC در سطوح ۰/۵٪ درصد سرخارگل و ۰/۱٪ و ۱٪ نعناع فلفلی، بیشترین مقدار را نشان داد. سطوح ۰/۵٪ نعناع فلفلی و ۱٪ بومادران و سرخارگل باعث افزایش معنی‌دار تعداد کل لوکوسیت‌ها و درصد نوتروفیل‌ها شد ($P\leq0.05$). سطوح ۰/۵٪ سرخارگل و ۱٪ هر سه گیاه باعث افزایش معنی‌دار لنفوسیت‌ها و کاهش معنی‌دار مونوسیت‌ها ($P\leq0.05$) شد. افزایش سطح ایمونوگلوبولین‌ها تنها در تیمار ۱٪ هر سه گیاه معنی‌دار بود ($P\leq0.05$). میزان C_3 در سطوح ۱٪ بومادران و نعناع فلفلی افزایش معنی‌داری نسبت به تیمار شاهد داشت ($P\leq0.05$). کلیه سطوح بومادران و نعناع فلفلی و سطوح ۰/۵٪ و ۱٪ سرخارگل باعث افزایش معنی‌دار غلظت لیزوزیم نسبت به تیمار شاهد شدند ($P\leq0.05$). نتایج نشان داد که عصاره خشک هر سه گیاه توان بهبود شاخص‌های خونی و پاسخ‌های ایمنی در ماهی کپور معمولی را دارند، اما در این بین، گیاه نعناع فلفلی با درصد پایین‌تر بازدهی بیشتری دارد.

کلمات کلیدی: گیاهان دارویی، ایمنی، خون‌شناسی، شاخص‌های رشد، کپور معمولی

***نویسنده مسئول:** soheilalinezhad47@gmail.com

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