Effects of *Zataria multiflora* and *Eucalyptus globolus* essential oils on haematological parameters and respiratory burst activity in *Cyprinus carpio*

Sheikhzadeh N.[1]; Soltani M.[1]; Ebrahimzadeh-Mousavi H. A.[2]; Shahbazian N.[2]; Norouzi M.[3]

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Abstract

The present study was undertaken to investigate the effects of *Zataria multiflora* and *Eucalyptus globolus* essential oils on some haematological parameters and respiratory burst activity in common carp (*Cyprinus carpio*). 260 fish (30±5g) were randomly distributed in 13 treatment groups; each one in three replicates and different doses of essential oils in 16-17°C were administrated. The fish were sampled on day 1, 2, 8, 15 and 22 after the 8-day trial. Haematological parameters (red blood cell count, haematocrit) and respiratory burst activity were then evaluated in all treatment groups. The results suggest that essential oils especially *Zataria multiflora* in dietary intake significantly enhanced respiratory burst activity of blood neutrophlis ($P< 0.05$). Meanwhile, essential oils had moderate effects on RBC and haematocrit. Significant increases in RBC and haematocrit levels were just noted in T_{11} treatment group ($P< 0.05$). This study indicates that dietary administration of *Zataria multiflora* and *Eucalyptus globolus* essential oils could be used to promote the health status of common carp during temperature stress.

Key words: *Zataria multiflora*, *Eucalyptus globolus*, Common carp, Temperature stress

1 -Department of clinical sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran.
1- Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
3- Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Tabriz, Tabriz, Iran.
*Corresponding author`s email: msoltani@ut.ac.ir

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Introduction

Common carp (*Cyprinus carpio*) is one of the main culturing species of fishes in Iran. Stressful environment renders the fish highly sensitive to different diseases. Low temperature is one of the main stresses in common carp that can even depress the immune responses. Use of immunostimulants is an advisable method to modulate the nonspecific immune system of fish during temperature stress (Bagni et al., 2005). Different kinds of immunostimulants are known but few of them are suitable for use in fish culture because of various disadvantages, such as high cost limited effectiveness.

On the other hand, a large number of plants have been used in traditional medicine for the treatment and control of several diseases. Two of such plants are *Zataria multiflora* and *Eucalyptus globolus*. *Zataria multiflora* is native to Iran, Pakistan and Afghanistan. The main compounds of this plant are thymol, carvacrol and para-cymene that contain 37.59, 33.65, and 7.72 % of the essential oil (Sharififar et al., 2007). *Zataria multiflora* essential oil also possesses a variety of biological activities such as anti inflamatory, antinociceptive and antimicrobial effects (Mansoori et al., 2002; Ramezani et al., 2004; Dakhili et al., 2006; Basti et al., 2007; Fazeli et al., 2007; ; Sharififar *et al.*, 2007; Khosravi et al., 2008, Choobkar et al., 2010). There is little evidence that this plant has immunostimulatory effects in laboratory animals. Shokri et al. (2006) observed an increase of the respiratory burst activity in BALB/C by injecting *Zataria multiflora* essential oil. In rabbits phagocytosis activity was also increased after injecting this essential oil subcutaneously (Khosravi et al., 2007). *Eucalyptus globolus* is native to the eastern part of Australia and grows in all continents. Leaves of this plant contain more than 70% cineol (Esser, 1993). There are some studies on the effects of *Eucalyptus globolus* against infectious and inflammatory diseases in human and animals (Vigo et al., 2004; Inouye et al., 2006; Cermelli et al., 2008) but few data are available concerning the influence of *Eucalyptus globolus* essential oil on the immune system. Serafino et al. (2008) showed the increased phagocytic activity of granulocytic/monocytic system in rats by *Eucalyptus globolus* essential oil. Few experiments showed that the antifungal and immunomodulatory effects of these two essential oils in rainbow trout (Sharifroohani, 2004; Sheikhzadeh et al., 2008; Soltani et al., 2010).

The present paper was undertaken to evaluate the effects of *Zataria multiflora* and *Eucalyptus globolus* on some parameters in common carp including respiratory burst activity, red blood cells and haematocrit during temperature stress.

Materials and methods

Animals and rearing conditions

Juveniles of common carp (30±5g) were obtained from a fish farm in Gilan province and kept in 300lit fiberglass tanks in Tehran Veterinary Faculty (Iran). The fish were acclimated with essential oil-free pellets (Chineh Company, Iran) for one month. Water temperature was 16-17°C during the experiment and was daily recorded..

Experimental design

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Fish were divided into 13 groups of 20 fish. In 6 groups fish were fed diets containing different doses of *Eucalyptus globolus* and *Zataria multiflora* essential oils (Barij essence company, Iran). Besides control groups, the remaining ones were administered different doses of essential oils by bath treatment for 30 min each day. The trial was conducted for 8 consecutive days. Different groups in this trial are summarized in table1:

Table1: Different treatment groups and administrations during the trial

Treatment group	Dose and method of treatment	Treatment group	Dose and method of treatment
T_1	Control	T_8	*Eg* oral 30ppm
T_2	*Eg* bath 30ppm	T_9	*Eg* oral 60ppm
T_3	*Eg* bath 60ppm	T_{10}	*Eg* oral 120ppm
T_4	*Eg* bath 120ppm	T_{11}	*Zm* oral 30ppm
T_5	*Zm* bath 7.5ppm	T_{12}	*Zm* oral 60ppm
T_6	*Zm* bath 15ppm	T_{13}	*Zm* oral 120ppm
T_7	*Zm* bath 30ppm		

Eg: Eucalyptus globolus; *Zm: Zataria multiflora*

Blood collection

On day 1, 2, 8, 15 and 22 after the end of an 8-day trial, 3 fish from each tank were cut from caudal peduncle and bled. The blood was then transferred immediately to a test tube containing EDTA 10% (as an anticoagulant) and shaken gently. The blood was used for determination of total erythrocytes, haematocrit and respiratory burst activity.

Total erythrocytes counting

For determining total red blood cells, RBC diluting fluid was used. Counting was done by mixing the blood with the diluting fluids. Cells counting was performed using a Neubauer`s counting chamber (Svobodova et al., 1996).

Haematocrit value

Microhematocrit capillary tubes were filled with blood and sealed. Then they were centrifuged for 7 min in 12000 rpm. Hematocrit values were read by a hematocrit reader (Svobodova et al., 1996).

Respiratory burst activity

The respiratory burst activity of neutrophils was studied by chemiluminescent assay following the method of Steele et al. (1991) and Shokri et al. (2006). Dextran (Pharmacia, Uppsala, Sweden) was added to blood in ratio of 2:1 (Dextran: Blood) and incubated at laboratory temperature for 30 min to allow the erythrocytes to sediment. Then the supernatant containing neutrophils was poured into a fresh tube.

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0.19ml of Ficoll (Sigma Chemical Co., St. Louis, USA) was added to the supernatant then re-suspended in 10ml of PBS with pH=7.2. Suspension was centrifuged at 1800×g for 10 min and supernatant was collected and brought to a final volume of 1 ml with PBS and neutrophils were counted. 500 µl of PBS, 200 µl of luminol (Sigma Chemical CO., Deisenhofen, Germany), 200 µl of phorbol 12-myristate 13-acetate (PMA) solution (Sigma Chemical Co., Deisenhofen, Germany) and 100 µl of neutrophil suspension were added to a special cuvett and its value was determined by the luminometer set (Biobarbitol 1251, Finland).

Statistical analysis

Kruscal-Wallis and Mann-Whitney tests for nonparametric analysis were run to compare different treatments using SPSS 16. The mean and standard errors were calculated for each treatment. The accepted level of significance was $P<0.05$.

Results

Total erythrocytes and haematocrit values of the experiment groups are shown in table 2 and 3 respectively. Different essential oils had no effects on the total RBC after treatment. However, there was significant increase in the total erythrocyte counts in T_{11} group on day 1, but on day 22 a significant decrease was shown in T_8 group compared with the control group ($P< 0.05$). Treating with either *Zataria multiflora* or *Eucalyptus globolus* essential oils had little effects on haematocrit. Addition of dietary *Zataria multiflora* at 30ppm (T_{11}) significantly increased the haematocrit value on day 15 but the lowest amounts were found in T_2 and T_5 treatment groups on day 8 compared to the control group ($P< 0.05$).

Data for respiratory burst activity of different treatment groups are given in table 4. These data showed that on day 1, significant increase in respiratory burst activity was just observed in T_{13} treatment group compared with the control group ($P< 0.05$). On day 2, this activity was significantly higher in all treatment groups than the control fish, with the highest value found in T_7 group ($P< 0.05$). Statistical analysis also showed that the activity was significantly increased on day 8 in T_3, T_4, T_5, T_{11}, T_{12} and T_{13} groups and highest activity was shown in T_{13} treatment group ($P< 0.05$). 15 days after trial, a significant increase in this activity was recorded in T_7, T_9, T_{11}, T_{12} and T_{13} groups ($P< 0.05$). On the last day of bleeding, activity level was significantly higher in T_5, T_6, T_{10}, T_{11}, T_{12} and T_{13} groups and peaked at 14.227± 0.61 mv in T_{11} ($P< 0.05$).

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Table2: Mean and standard errors for red blood cell counts analyzed in control and different groups

Treatment group	Bleeding day				
	1	**2**	**8**	**15**	**22**
T_1	868330± 26884	885000± 39475	870000± 42268	903330± 49441	846670± 19944
T_2	863330± 3333	886670± 46666	876670± 12018	850000± 65574	950000± 132288
T_3	766670± 37564	870000± 55075	866670± 33333	823330± 17638	870000± 17320
T_4	896670± 27284	843330± 31797	893330± 17638	950000± 83864	846670± 81103
T_5	933330± 33333	1000000± 104083	9166670± 61191	996670± 31797	866670± 56075
T_6	850000± 66583	916670± 67659	886670± 13333	903330± 42557	976670± 23333
T_7	943330± 29627	903330± 42557	996670± 31797	973330± 81921	793330± 58118
T_8	926670± 37118	846670± 24037	910000± 49328	906670± 57831	750000± 28867*
T_9	826670± 42557	853330± 24037	923330± 721188	900000± 11547	850000± 50000
T_{10}	866670± 68879	883330± 27284	806670± 49777	960000± 37859	810000± 20816
$_{11}$	1046700± 76883*	833330± 33333	900000± 57735	9330000± 35118	833333± 37564
T_{12}	830000± 32145	846670± 24037	850000± 56862	823330± 46308	850000± 28867
T_{13}	940000± 34641	910000± 95393	920000± 70000	870000± 36055	870000± 65574

* Significant difference (P<0.05) compared to the respective control group

Table3: Mean and standard errors for haematocrit values analyzed in control and different groups

Treatment group	Bleeding day				
	1	**2**	**8**	**15**	**22**
T_1	26.5± 1.43	24.17± 0.79	27± 1.29	24.83± 0.54	26.83± 1.49
T_2	27± 2.08	24± 2	20.66± 2.02*	25± 3	28.33± 1.66
T_3	27± 1.15	26.66± 1.20	28± 1.53	25.66± 1.20	24.33± 1.46
T_4	26.66± 0.88	226.33± 1.33	23.66± 0.66	24.66± 1.20	25.33± 1.45
T_5	25± 0.58	25.33± 0.88	22.33± 0.33*	24.33± 0.88	25.33± 0.88
T_6	29.33± 1.76	25.33± 0.66	26± 1.52	23.66± 0.66	27.66± 1.45
T_7	25.33± 2.40	22.33± 1.20	27± 1.73	25.33± 0.88	27.66± 2.33
T_8	23.66± 0.88	25.33± 2.18	30± 1.52	26.66± 2.72	31.66± 1.33
T_9	28.33±0.88	26.33± 2.40	28.33± 2.02	25± 1.52	24.66± 1.66
T_{10}	26± 1.53	23.33± 0.88	24.66± 2.02	26± 1.15	25.33± 1.45
T_{11}	24± 33.05	24.67± 0.33	26.33± 0.88	28.33± 0.88*	29± 1
T_{12}	30± 0.58	24± 0.58	27.33± 1.76	25.33± 0.88	25.33± 1.45
T_{13}	25± 1.73	27± 1.522	24.66± 0.33	23.33± 1.20	24.66± 0.66

*Significant difference (P<0.05) compared to the respective control group

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Table4: Mean and standard errors for respiratory burst activity analyzed in control and different groups

Treatment group	Bleeding day				
	1	2	8	15	22
T_1	13.92± 2.14	8.39± 0.53*	9.02± 0.65	9.27± 0.79	8.77± 0.57
T_2	11.20± 0.50	12.57± 1.14*	9.90± 0.18	11.67± 0.95	10.63± 0.78
T_3	14.31± 1.96	13.45± 0.40*	12.03± 0.49*	11.40± 0.20	10.90± 0.74
T_4	13.05± 1.07	11.24± 0.50*	11.72± 1.04*	10.67± 0.30	10.70± 0.53
T_5	16.84± 1.40	12.84± 0.30*	11.57± 0.75*	11.45± 0.33	12.24± 1*
T_6	17.75± 2	13.71± 1.03*	11.06± 0.83	11± 0.30	12.05± 0.55*
T_7	15.64± 1.39	15.38± 0.51*	11.42± 0.89	13.10± 0.15*	11.22± 1.08
T_8	21.46± 1.64	11.66± 0.91*	10.37± 0.97	9.80± 0.64	9.92± 0.51
T_9	11.73± 1.58	11.86± 1.07*	11.39± 0.42	13.15± 0.18*	9.43± 0.88
T_{10}	20.19± 1.24	13± 0.16*	11.10± 0.63	12.84± 0.90	12.89± 0.40*
T_{11}	19.31± 11.08	14.39± 1.24*	13.38± 1.10*	15.21± 0.78*	14.27± 0.61*
T_{12}	16.11± 1	14.30± 1.37*	12.97± 0.80*	13.49± 1.63*	12.64± 0.76*
T_{13}	21.79± 0.97*	14.55± 0.40*	13.99± 0.46*	13.21± 1.46*	12.54± 0.78*

* Significant difference (P<0.05) compared to the respective control group

Discussion

Farmed fishes are inevitably subjected to different kinds of stress during the period of culture so a practical immunostimulant can upregulate the nonspecific immune system just before or during a stressful condition to protect fish and reduce the rate of mortality.

In the previous studies, effects of *Eucalyptus globolus* and *Zataria multiflora* essential oils on rainbow trout immunity showed the augmentation of some immunological factors such as antibody titers, total white blood cells and serum bactericidal activity in some doses especially in dietary intakes (Sheikhzadeh et al., 2008; Soltani et al., 2010).

In the present study we investigated some haematological parameters including total red blood cell count and haematocrit besides the respiratory burst activity by chemiluminescent assay under low temperature. There was no significant difference in the total red blood cell count and haematocrit value among the groups after receiving the essential oils but a significant increase was just observed in T_{11} group on some days of bleeding. This result is supported by other studies which found that there was no significant increase in these parameters when fish were treated with different immunostimulants like ribonucleic acid, chitin and lactoferrin (Choudhury et al., 2005; Esteban et al., 2005).During a period of intense oxygen consumption that is called respiratory burst, activated phagocytes are able to produce superoxide onions and its reactive derivatives. These reactive oxygen species are toxic for fish bacterial pathogens (Choudhury et al., 2005). Different components, including levamisole, glucan and yeast RNA are known to stimulate phagocytes (Choudhury et al., 2005). In the present study, the respiratory burst activity was examined by chemiluminescent assay. In common carp these essential oils in all treatment groups enhanced the respiratory burst activity after 2 days of the 8-day trial but in other days enhancement was more noted in dietary intake of essential oils especially groups which received *Zataria multiflora* essential oil. This is in agreement with the results of Shokri et al. (2006) which showed the significant augmentation in the respiratory burst

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activity in mice injected with *Zataria multiflora* essential oil peritoneally.

It is noteworthy to mention that response of common carp can be diminished when they are kept at low temperature. For example, in the healthy carp under optimum temperature erythrocyte count ranges from 1100000-1800000 per ml (Svobodova et al., 1996). As it was shown in this study, total RBC counts in all treatment groups besides control group were below 1046000 that are less than the normal range but the essential oils in dietary administration had the potential to activate the responses and make the ranges near to normal ones in optimum temperature. In conclusion, the results of this study suggested the efficacy of these essential oils to augment some immunological and haematological parameters in common carp especially under condition of immunodepression related to environmental stress.

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