Dietary thyme essential oil (*Thymus vulgaris*) changes serum stress markers, enzyme activity, and hematological parameters in gibel carp (*Carassius auratus gibelio*) exposed to Silver Nanoparticles

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Abstract

Gibel carp (*Carassius auratus gibelio*) juveniles with an average weight of 8.73± 2.1 g were fed a basal diet (control) and three experimental diets (T200, T400 and T800), containing 200, 400 and 800 mgkg^{-1}, respectively of thyme essential oil (*Thymus vulgaris*) for six weeks. After six weeks feeding trial, both control and thyme essential oil-added feed groups were exposed to sub-acute toxicity of silver nanoparticles (Ag-NPs) for a period of 96 h. At 96 h after exposure, the number of erythrocytes (RBC), leucocyte (WBC), hematocrit (Hct), hemoglobin (Hb), the differential leukocyte count in blood and alanine aminotransferase (ALT), aspartate aminotransferase (AST) activity, HDL-cholesterol, LDL-cholesterol, cortisol and lactate in serum were measured. The results revealed feeding a thyme essential oil diet significantly conferred resistance to oxidative stress with sub-acute toxicity of Ag-NPs. Thyme essential oil enhanced the RBC and WBC count, hematocrit and hemoglobin content, at both low (T400) and high doses (T800). Biochemical analysis showed that serum ALT and AST activities, and LDL-cholesterol in the thyme essential oil-added feed groups were significantly reduced with increasing dietary thyme essential oil. Plasma HDL-cholesterol was significantly elevated by a high-dose of dietary thyme essential oil (T800). Furthermore, cortisol and lactate levels in serum significantly decreased throughout the thyme essential oil-added feed groups compared to the control. In summary, the use of thyme essential oil at 400 and 800 mgkg^{-1}, as dietary supplements, has potential to decrease oxidative stress of gibel carp providing resistance to non-fatal effects of pollutant by Ag-NPs.

Keywords: Silver nanoparticles, Gibel carp, Thyme essential oil, Plasma electrolytes, Hematological parameters

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Introduction

With regard to increasing environmental contaminants and its stimulating effects on oxidative stress, improving the antioxidative status of fish seems to be necessary (Welker and Congleton, 2005). In order to improve health conditions and resistance to stressor agents in the farming of aquatic organism, several alternatives such as use of phytochemicals, antioxidants, probiotics and immunostimulants have been proposed. The growing interest in medicinal plants including herbs, spices, seaweeds, herbal extracted compounds, and commercial plant-derived products has increased world-wide because they are easy to prepare, cheap, and have few side effects on animals and the environment (Van Hai, 2015). Plants or their byproducts contain several antioxidant and antimicrobial properties due to the presence of alkaloids, flavanoids, pigments, phenolics, terpenoids, steroids and essential oils, and therefore, they are prescribed as chemotherapeutic molecules in aquaculture (Citarasu *et al.,* 2002; Sivaram *et al.*, 2004; Talpur *et al.*, 2013). A diverse range of herbs is used in aquaculture including almond (Chitmanat *et al.,* 2005), ginger (Punitha *et al.,* 2008; Talpur *et al.*, 2013), seaweeds (Immanuel *et al.*, 2010), basil flowers (Peraza-Gómez *et al.*, 2011), cinnamon (Ahmad *et al.*, 2011), garlic (Nya and Austin, 2011), thyme (Yılmaz *et al.*, 2012), onion (Cho and Lee, 2012), green tea (Hwang *et al.*, 2013), and rosemary (Hernández *et al.*, 2015a). They are either available in a solid, dried or ground form or as extracts or essential oils. Aromatic/medicinal plants and their essential oils which are very rich in polyphenols, have proven beneficial health effects. Essential oils that are also known as volatile or ethereal oils are concentrated hydrophobic liquids containing the volatile aroma compounds of plants. The main groups of constituents found in essential oils include alcohols, aldehydes, esters, ethers, ketones, phenols, and terpenes. However, most people know of their use in medicine based on important biological activities, such as antimicrobial activity, anti-oxidant activity, anti-inflammatory activity, cytotoxic activity etc. (Kamatou *et al.*, 2008; Adel *et al*,, 2016). This class of feed additives has recently gained increased interest in animal feeding. Most studies on application of essential oils in animal nutrition have been conducted in swine and poultry, however, there is increasing evidence that the application of essential oils can also be beneficial in aqua species (Abutbul *et al.*, 2004; Cardozo *et al.*, 2008; Rojas, 2007; Hernández *et al.*, 2014, 2015b; Haghighi *et al.*, 2017). Thyme oil has been tested as a feed additive in fish and has been demonstrated to have strong antimicrobial and antioxidant activity due to its very high contents of thymol p-cymene, carvacrol, eugenol and 4-

allylphenol (Lee *et al.,* 2005; Rota *et al.,* 2008).

Silver nanoparticles (AgNPs) are an important class of nanomaterials and currently attract considerable interest from both academia and industry because of their interesting and diverse properties. It is widely known that nanoparticles may cause potential adverse effects on organisms, including aquatic species (Bilberg *et al.,* 2012), human (Haase *et al.,* 2011) and mammals (Muller *et al.,* 2005). Because many of the aquatic species are more sensitive to silver than mammals and humans the data on effects of silver on aquatic organisms is of eminent importance. Therefore, it is important to develop an understanding of the effects of nanoparticles on aquatic organisms. It has been mentioned that silver nanoparticles (Ag-NPs) have an adverse impact on aquatic organisms, and cause oxidative stress, cellular apoptosis, changes in gene expression, chromosomal aberrations, and aneuploidy during early life stages and adulthood (Asharani *et al.,* 2008; Wise *et al.,* 2010; Wu *et al.,* 2010). Despite the dramatic increase in the use of Ag-NPs in products such as textiles, paints, pharmaceuticals, food production and cosmetic products (Chen and Schluesener, 2008), little data are available on their potential harmful effects on ecosystems (Lovern *et al.,* 2007). Ag-NPs can easily leak into waste water during washing, thus endangering aquatic organisms in lakes and streams (Benn and Westerhoff, 2008). It has been shown that lower doses of nanoparticles also cause respiratory toxicity in rats and adverse effects in human macrophages (Muller *et al.,* 2005; Haase *et al.,* 2011).

Gibel carp also known as goldfish, is one of the most popular freshwater ornamental species in the world due to its varieties with attractive body shape and skin color (Zhou *et al.,* 2001). Furthermore, this species is widely used as a laboratory model organism for biomedical research, such as in developmental genetics, neurophysiology and ecotoxicology (Trudeau, 1997; Spanò *et al.,* 2004; Mimeault *et al.,* 2005). However, because gibel carp is a laboratory model, we chose it for investigating the environmental effects of nano-materials. The present study aimed at evaluating whether the dietary thyme essential oil, by supplementing a basal diet with different doses, would affect the biochemical traits of the blood and hematological of gibel carp exposed to sub-acute toxicity of Ag-NPs.

Materials and methods

Ethics

All procedures involving animals were conducted in accordance with the Iranian law on experimental animals and were approved by the Ethical Committee for Animal Experiments of Iran's Veterinary Organization. Efforts were made to minimize suffering.

Fish and maintenance conditions

A total of 300 gibel carp (*C. auratus gibelio*) juveniles with an average body weight of 8.73±2.1 g and 6.45±0.7 cm standard length were obtained from a local commercial distributor (Kurdistan Province, Sanandaj, Iran) and distributed equally into three 500 L rectangular fiberglass tanks (127×76×127 cm) in the aquarium facilities of the fish biology lab at the University of Kurdistan, Iran with Sanandaj City tap water. The animals were in healthy condition and devoid of any apparent malformations. The fish were acclimatized to laboratory conditions at ambient temperature (22±2°C) with dissolved oxygen content of 7±02 mgL^{-1}, ammonia 0.12±0.01 mgL^{-1}, nitrites 0.05±0.04 mgL^{-1}, total hardness 184.6±15.1 mgL^{-1}, pH 8.2±0.3 and 12 h dark/12 h light cycle (12L:12D). The freshwater used to culture the fish was dechlorinated and continuously aerated. Fish were maintained under these conditions for over a 2-week period to recover from stress. During the 2 weeks of acclimatization, fish were fed a basal diet at 3–5% of body weight twice a day, one at 8:00 in the morning, the other at 17:00 hours in the afternoon.

Experimental design and diets

Fish were randomly divided into four groups (75 fish each) i.e., a basal diet (control) and three experimental diets (T200, T400 and T800), containing 200, 400 and 800 $mgkg^{-1}$, respectively of thyme essential oil (*T. vulgaris*, thymol chemotype; 57.7% thymol, 18.7% p-cimeno, 2.8% carvacrol; MONIN, France). Each group of 75 fish was again divided into three equal subgroups and maintained in 70-L aquariums (58×32×42.5cm) containing 50-L dechlorinated tap water. Thyme essential oil was mixed directly with fish feed contents and these isoprotein and isoenergetic diets were formulated in accordance with gibel carp nutrient requirements (Table 1). The feeding trial was conducted for six weeks prior to the exposure test on 3–5% of body weight, with the daily ration being subdivided into two equal parts and fed twice a day.

Sub-acute exposure test

Ag-NPs with average particle size of 61 nm were purchased from Nanocid® (Tehran, Iran) in a suspension form. A stock solution of Ag-NPs was prepared at 10 mgL^{-1} and working solution (0.04 μgmL^{-1}) was obtained by diluting the stock solution with amounts of deionized (DI) water. Immediately after six weeks feeding trial, Both control and thyme essential oil-added feed groups were exposed to sub-acute toxicity of Ag-NPs (0.04 μgmL^{-1}) in 70-L aquariums containing 50-L dechlorinated tap water for a period of 96 h, according to the method described by Shaluei *et al.* (2013). The fish were not fed during the experiment in order to maintain constant exposure concentrations, given that nanoparticles might adhere to food particles and feces (Lead and Wilkinson, 2006).

Table 1: Ingredients and proximate composition of the basal diet.

Ingredients	Content (g/kg)
Fish meal	290
Soybean meal	290
Corn meal	200
Clupen fish oil	20
Wheat flour	166
Lime	5
Methionine	2
Lysine	2
Dicalcium phosphate	5
Salt	5
Vitamin premix[a]	7.5
Mineral premix[a]	7.5
Proximate composition	
Dry matter (%)	89.21 ± 1.33
Crude protein (%)	33.71 ± 0.20
Crude fat (%)	8.41 ± 0.28
Ash (%)	9.22 ± 0.47
Fiber (%)	2.92 ± 0.06

The water in the aquaria was changed daily and freshly prepared working solution was added to maintain the concentration of Ag-NPs at a constant level (0.04 µgmL^{-1}).

Sampling and analysis

At 96 h after exposure, blood samples were collected from both control and thyme essential oil-added feed groups. Fish were gently captured from each tank using appropriate dip net and quickly transferred to an anesthetic bath (clove solution, 100 ppm). Blood samples were drawn from caudal vein using a medical syringe. Each fish blood sample was divided into two parts. One was heparinized immediately for analysis of erythrocytes (RBC), leucocyte (WBC), hematocrit (Hct), hemoglobin (Hb), and the differential leukocyte count (leukogram). The other half was collected in the non-heparinized plastic tubes over less than 2 min per sample. The samples were kept to clot at 4°C for 2 h then centrifuged (3,000rpm, 15 min) for serum separation. The serum samples were stored at –20°C for further analyses.

RBC (×10^6) and WBC (×10^3) counts were determined microscopically by hemocytometry (Blaxhall and Daisley, 1973; Houston, 1990), using an improved Neubauer chamber after diluting blood samples with Dacie’s solution. Hematocrit (Hct) was determined with microcentrifuge method at 3500× g for 10 min of standard heparinized microhematocrit capillary tubes (75 mm long with an inner bore of 1.2 mm) and expressed as a percentage (Blaxhall and Daisley, 1973). Hemoglobin (Hb: g/dL) was measured spectrophotometrically at 540 nm on the basis of cyanmethemoglobin procedure (Drabkin, 1945). Differential leucocyte count was determined in blood smears from one drop of homogenized fresh blood, stained with Wright-Giemsa (WG) fluid for 8 min, and washed twice in distilled water for 1 min, then air-dried. The stained smears were examined under oil immersion using a high power light microscope (Olympus DP 12, Tokyo, Japan; 100 × magnifications) and the leucocytes identified according to Watson *et al.* (1963). At least 200 leucocytes were counted on each slide and classified as lymphocyte, monocyte, neutrophil and eosinophil.

The plasma cortisol was determined by ELISA method (Barry *et al.*, 1993) using commercial kit (Demeditec, Cat. -No. DE1887; Germany) and plasma cortisol was expressed as $ngmL^{-1}$. Intra- and inter-assay variances for the cortisol were 5.6 and 7.7%, respectively, with a minimum detection limit of 2.5 $ngmL^{-1}$.

Serum alanine aminotransferase (ALT; UV IFCC kinetic method), aspartate aminotransferase (AST; UV IFCC kinetic method) activity, HDL-cholesterol (direct colorimetric enzymatic method), LDL-cholesterol (liquid colorimetric enzymatic method), and lactate (direct colorimetric enzymatic method) level were determined using commercially available reagent kits (Pars Azmun Co. Ltd., Tehran, Iran) and a double-beam UV-VIS spectrophotometer coupled to a thermo system (SECOMA, NorthStar, Scientific Ltd; UK). Each measurement was performed in triplicate.

Statistical analyses

Values were expressed as mean±standard error of mean. Data were analyzed using one-way ANOVA after testing the homogeneity of variances with the Levene test and the comparison of the mean values was done by using Duncan multiple range tests. Differences were measured and considered to be statistically significant at $p<0.05$. All statistical analyses were conducted using SPSS for Windows, Version 16.0 (SPSS Inc., Chicago, IL, USA).

Results

Throughout the feeding trial and sub-acute exposure test, mortality never exceeded an average of 3%; in both control and thyme essential oil-added feed groups. All diets were readily accepted by the fish. The RBC and WBC counts, hemoglobin content, hematocrit, and the differential leukocyte count (leukogram) of different groups are given in Fig. 1.

Marked hematological and biochemical changes were evident in the control fish at 96 h after sub-acute exposure test compared to the thyme essential oil-added feed groups.

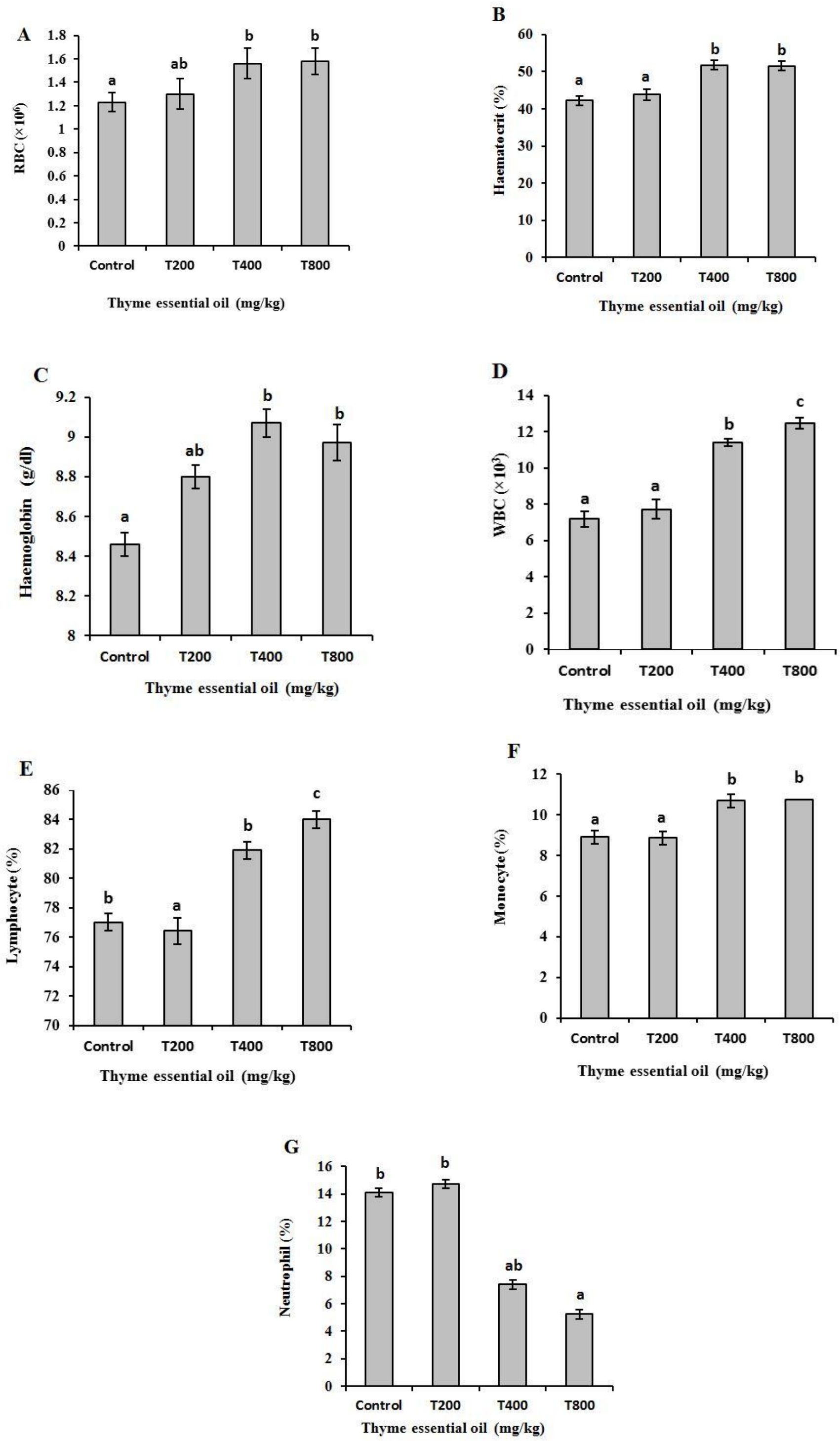

Figure 1: Effect of dietary thyme essential oil at different levels on haematological values RBC (A), hematocrit (B), hemoglobin (C), WBC (D), lymphocyte (E), monocyte (F), and neutrophil (G) of gibel carp (*Carassius auratus gibelio*) after exposure to sub-acute toxicity of Ag-NPs. Data expressed as mean±SE. Different letters indicate values significantly different (*p*<0.05) from each other.

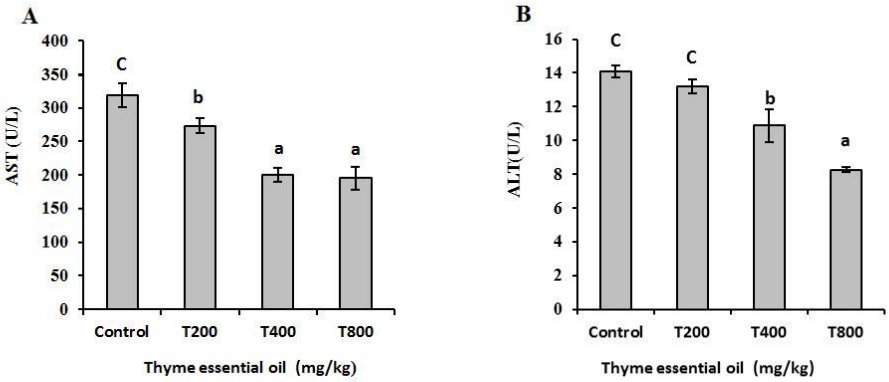

Figure 2: Effect of dietary thyme essential oil at different levels on serum AST (A) and AST (B) levels of gibel carp (*Carassius auratus gibelio*) after exposure to sub-acute toxicity of Ag-NPs. Data expressed as mean±SE. Different letters indicate values significantly different (*p*<0.05) from each other.

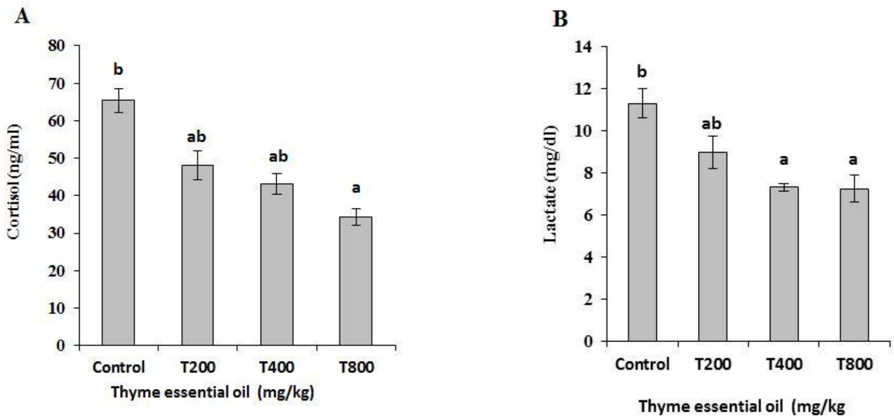

Figure 3: Effect of dietary thyme essential oil at different levels on serum stress markers cortisol (A) lactate (B) levels of gibel carp (*Carassius auratus gibelio*) after exposure to sub-acute toxicity of Ag-NPs. Data expressed as mean±SE. Different letters indicate values significantly different (*p*<0.05) from each other.

The control fish exposed to Ag-NPs, showed significantly lower RBC count, heematocrit and hemoglobin content ($p<0.05$) compared to the thyme essential oil-added feed groups (Fig. 1A, B and C). Thyme essential oil enhanced the RBC count, hematocrit and hemoglobin content, at both low (T400) and high doses (T800). The WBC count started to increase with increasing dietary thyme essential oil (Fig. 1D).

Fish fed with thyme essential oil-supplemented diets were affected in their proportions of lymphocyte, monocyte, and neutrophil compared with the control group. There was significantly higher number of neutrophil in the control fish and T200 exposed to Ag-NPs ($p<0.05$) compared to the high dose of thyme essential oil-added feed groups (Fig. 1G). Whereas the number of lymphocyte and monocyte showed a progressive decline in the T200 and control fish (*p*<0.05) compared to the high dose of thyme essential oil-added feed groups (Fig. 1E and F). Eosinophils were not observed in any of the blood samples.

A 96 h sub-acute exposure test of Ag-NPs caused the plasma AST and ALT activities to significantly increase (*p*<0.05) in the control group compared to the thyme essential oil-added feed groups (Fig. 2A and B). However, plasma AST and ALT activities started to decrease with increasing dietary thyme essential oil.

Furthermore, lactate levels in T400 and T800 groups significantly decreased (*p*<0.05) compared to the control and T200 groups (Fig. 3B). Sub-acute exposure test of Ag-NPs significantly increased plasma LDL-cholesterol (*p*<0.05) in the control fish (Fig. 4B). However, this parameter was significantly reduced by high-dose dietary thyme essential oil (T800). Moreover, enriched diets with higher dose of thyme essential oil (T800) elevated HDL-cholesterol compared to the other groups (Fig. 4A).

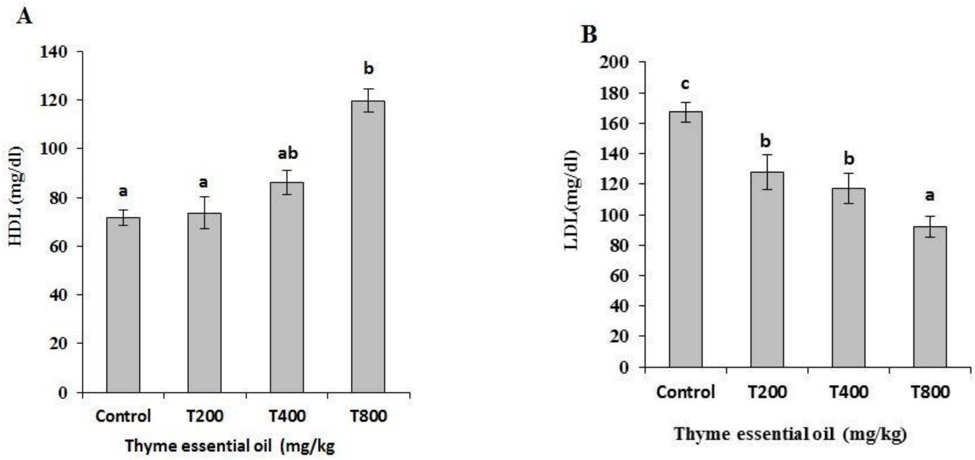

Figure 4: Effect of dietary thyme essential oil at different levels on serum biochemical HDL (A) LDL (B) levels of gibel carp (*Carassius auratus gibelio*) after exposure to sub-acute toxicity of Ag-NPs. Data expressed as mean±SE. Different letters indicate values significantly different (*p*<0.05) from each other.

Discussion

In aquaculture fish are consistently affected by various stress factors such as overcrowding, handling, temperature changes, poor nutritional status, and environmental pollutants. Environmental stressors have great impact on the axis of hypothalamus–pituitary–interrenal. Axis of animal will be continuously stimulated so as to cause higher levels of serum stress markers, and may impair natural host defense systems. The concept of Ag-NPs either leaching or being released into aquatic environments is of particular concern considering the many years of research showing that Ag-NPs is highly toxic to various freshwater aquatic species (Lee *et al.,* 2007; Asharani *et al.,* 2008; Griffitt *et al.,* 2008; Laban *et al.,* 2010). We found notable changes of serum stress markers, enzyme activity, and some hematological parameters in gibel carp in response to Ag-NPs exposure test.

Hematological parameters are typically used to assay the health status and monitoring the stress responses including those due to exposure to toxicants (McLeay and Gordon, 1997; Orun *et al.,* 2005; Ates *et al.,* 2008). In the present study, RBC count, hemoglobin content, and hematocrit showed increasing trends in concert with increasing doses of thyme essential oil in the diets, which is in conformity with the finding of Kumar *et al.* (2013) in goldfish fed a diet containing azadirachtin. Moreover, in accordance with our results, Harikrishnan *et al.* (2010) showed a significant increase in RBC count, hemoglobin content, and hematocrit; following feeding goldfish with 200 and 800 $mgkg^{-1}$ of mixed herbal extract enriched diets. Decreased RBC count, hemoglobin content, and hematocrit of control fish in the present study indicate that exposure test of Ag-NPs might destroy or cause lysing of RBCs due to toxicant stress leading fish to anemia. Similarly, decreases in RBC count were also reported in fish exposed to sublethal doses of nitrite (Ciji *et al.,* 2012), manganese sulphate (Agrawal and Srivastava, 1980), subacute toxicity of Ag-NPs (Shaluei *et al.,* 2013), and other toxicants (Dick and Dixon, 1985; Ates *et al.,* 2008; Vani *et al.,* 2012;). Hemostasis observed in Japanese medaka embryo when exposed to Ag-NP solutions at different concentrations (Wu *et al.,* 2010). It has been reported that Ag-NPs are cytotoxic to rainbow trout erythrocytes (Massarsky *et al.,* 2014). However, the precise mechanism whereby silver nanoparticles exert their toxicity to fish hematological parameters is unknown to our knowledge.

Due to the role of WBC in non-specific or innate immunity (Kumar *et al.,* 2007), increase in the WBC count and its functions is quite likely to result in an enhancement of the non-specific defense. Many studies have shown that immunostimulants, such as tuftsin, *C. versicolor* polysaccharides, azadirachtin and ginger elevated the WBC count of fish (Misra *et al.,* 2006; Nya and Austin, 2009; Kumar *et al.,* 2013; Wu *et al.,* 2013). Our results indicate that, the WBC count increased in T400 and T800 groups compared to the control. The increasing trend in WBC count could be related to a stimulation of the immune system with thyme essential oil due to phenolic compounds such as eugenol (2-methoxy-4-(2-propenyl) phenol), thymol and carvacrol present in essential oils. Similar to the present study, Wu *et al.* (2013) reported that supplementary diets with suitable doses of *Coriolus versicolor* polysaccharides enhanced the WBC count in crucian carp when challenged with *Aeromonas* *hydrophila*. Additionally, dietary thyme essential oil for a period of 30 days, improved the survival of Atlantic salmon when challenged with *Saprolegnia parasitica* (Rojas, 2007). In the current study, lymphocyte and monocyte numbers declined in the T200 and control fish in response to exposure test stress. However, these parameters showed a progressive increase with increasing doses of thyme essential oil in the diets, revealing an effect of thyme essential oil on lymphocyte and monocyte aggregates. Nya and Austin (2009) reported similar results in the proliferation of lymphocytes and enhanced phagocytic activity of rainbow trout fed with ginger. Inclusion of thyme essential oil in the feed, exhibited an increase in the occurrence of lymphocyte aggregates in the lamina propria of intestines in gilthead seabream (Hernández *et al.*, 2015b). Our findings indicate that, the decrease in the WBC count, lymphocyte and monocyte numbers observed in the control fish, may be associated with a decrease in nonspecific immunity of the fish due to exposure test and enhanced susceptibility to disease, thus enriched diets with essential oils have beneficial effects on fish health and enhance their immune system.

Plasma enzyme activity provides important information on the functional state of different organs as their activities might be altered by disease, chemical exposure to toxic or stress conditions. ALT and AST are two of the important enzymes in the hepatocytes as the indicators of hepatotoxicity. Increased activity of ALT and AST in serum is associated with hepatocytes or tissue damage caused by environmental pollution and leaching of these enzymes in blood. Machala *et al.* (1997) reported that even low-levels of organic contamination lead to increased hepatocytes enzymes activities in fish. In the present study, sub-acute exposure test of Ag-NPs caused a dramatic increase in the plasma AST and ALT activities in the control fish. This is in accordance with findings of Louei Monfared *et al.* (2015) that exposure to nanosilver resulted in a significant increase in the activities of plasma AST and ALT in rainbow trout. However, our findings showed that the plasma AST and ALT activities started to show a dose-dependent decrease in response to dietary thyme essential oil. Study by Salahel-deen and Rogers (1993) showed serum AST and ALT activities increased significantly in plasma, muscle, and liver of grass carp exposed to diquat herbicide. Moreover, Fırat *et al.* (2011) reported serum ALT and AST activities of Nile tilapia increased in response to copper (Cu), lead Pb, and cypermethrin (CYP) exposures during 4 and 21 days. Certain herbs have been reported to be able to protect liver and alter serum ALT and AST activates in fish. Rao *et al.* (2006) reported, elevated levels of serum ALT and AST were brought back to normal by *Achyranthes aspera* in *Labeo rohita* infected with *Aeromonas hydrophila*.

Serum AST and ALT activities decreased significantly in Nile tilapia fed with various levels of garlic (Shalaby *et al.*, 2006). Additionally, significant reduction in serum AST and ALT activities was observed following onion administration in *Clarias lazera* diet (Al-Salahy, 2002). These results can be attributed to flavonoids and phenolic compounds present in the essential oils, which may cause stabilized cell membrane and protect the hepatocytes against deleterious agents and toxic compounds to cells that is reflected in alteration of serum enzymes. Another way thyme essential oil may exert antioxidative effects is by increasing the bioavailability of vitamin C and E in fish body, thus increasing the body's natural antioxidant.

Plasma cortisol and lactate are general indices of stress status induced by various external and ecological stressors. The rise of plasma cortisol content can be considered as a primary response to stressors such as biological agents or environmental conditions. Lactate is produced by anaerobic metabolism in the white muscle under conditions of hypoxia (Driedzic and Kiceniuk, 1976). Stress-induced lactate production can cause reductions in flesh quality by lowering tissue pH (Elvevoll *et al.*, 1996). In the present study, sub-acute exposure test of Ag-NPs caused an increase in plasma cortisol levels in both control and low doses of thyme essential oil-added feed groups (T200 and T400). However, the plasma cortisol level apparently decreased in the test group supplemented with 800 $mgkg^{-1}$ thyme essential oil. It indicated that high dose of dietary thyme essential oil could alleviate cortisol load caused by sub-acute exposure test of Ag-NPs and indirectly raise the capability to resist environmental contaminants. Cortisol elevation was reported in many studies after exposure of fish to a variety of toxic substances (Bennett and Wolke, 1987; Fırat *et al.*, 2011; Shaluei *et al.*, 2013). Our findings showed, lactate level significantly raised in the control fish suggesting that the fish entered oxygen debt after exposure test, a result of meeting more energy demands by anaerobic respiration in the white muscle (Black, 1957). Serum lactate in shark, chinook salmon, golden perch and goldfish raised quickly when they are subjected to different stressor agents (Cliff and Thurman, 1984; Barton *et al.*, 1986; Carragher and Rees, 1994; Sinha *et al.*, 2012). Similarly, stress recovery in the Japanese flounder was reported by dietary medicinal herbs mixture (Ji *et al.*, 2007). Moreover, Xie *et al.* (2008) reported, 1%–2.0% anthraquinone extract supplemented in a basal diet decreased blood cortisol levels of the common carp fingerlings were exposed to crowding stress.

HDL-cholesterol and LDL-cholesterol are important indicators for lipid metabolism. Oxidative stress and severe lipid peroxidation have been observed in fish brain tissue upon exposure to nanomaterials (Oberdörster, 2004). Lipid peroxidation can be repealed by increased HDL activity, which prevents LDL oxidation, eventually reducing serum lipids (Mackness *et al.*, 1993; Cesar *et al.*, 2010). Massarsky *et al.* (2014) showed that Ag-NPs induced lipid peroxidation by generating reactive oxygen species (ROS) extracellularly or within close proximity to the cell membrane in rainbow trout hepatocytes. Significant antioxidative properties are well described for essential oils especially in the case of the phenolic compounds derived from Labiatae plant family (Craig, 1999; Burt, 2004; Wei and Shibamoto, 2007). On the other hand, dietary herbs may promote cellular lipid and fatty acid utilization (Ji *et al.*, 2007). The present study clearly showed that sub-acute exposure test of Ag-NPs caused lower serum HDL-cholesterol and higher LDL-cholesterol levels in both control and low dose of thyme essential oil-added feed groups (T200 and T400). It is proved that oxidative stress could interfere in the gastrointestine tract by interruption of digestibility and absorption of nutrients in intestine. However, LDL-cholesterol was significantly reduced by a high-dose of dietary thyme essential oil (T800). On the other hand, enriched diets with a higher dose of thyme essential oil (T800) elevated HDL-cholesterol compared to the other groups. This is an indication that dietary thyme essential oil has the ability to enhance HDL activity, which inhibit LDL activity, and subsequently reduce serum lipid. Similar to our study, dietary medicinal herbs mixture and dietary *Aloe vera* elevated HDL-cholesterol in Japanese flounder and GIFT-tilapia, respectively (Ji *et al.*, 2007; Gabriel *et al.*, 2015). On the contrary, a study by Chen *et al.* (2014) showed, that supplementary diets with a higher dose of chitosan (20 000 $mgkg^{-1}$) significantly reduced plasma HDL-cholesterol in gibel carp. Additionally, HDL-cholesterol level in the plasma of black rockfish was not significantly affected by the dietary green tea ethanol extracts (Hwang *et al.*, 2013). Our findings indicate that the antioxidant property of many essential oil compounds may be assumed to protect body lipids from oxidation like antioxidants usually added to diets. Thus, the use of essential oils as dietary feed additives may clearly improve quality of animal derived products and presumably the animals' cardiovascular health too.

Sustainable aquaculture development requires the use of safe and effective solutions to overcome the challenges in this industry. There is increasing evidence that natural products such as essential oils could have applications in aquaculture, to achieve a good feed efficiency, growth promotion, animal health, and increase stress resistance. An important finding from the present study was that even low-levels of nano-materials at non-fatal dose may have potentially toxic effects and lead to changes in health status of fish. It is concluded that, the use of thyme essential oils, as dietary supplements, can improve the innate defense of gibel carp in providing resistance to non-fatal effects of pollutants by Ag-NPs. Although the precise mechanisms of essential oils are not clear further investigations are necessary especially in molecular mechanism levels for the effective use of thyme essential oil in aquaculture.

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