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EVALUATION OF THE IMMUNOMODULATORY EFFECTS OF SILYMARIN EXTRACT (*SILYBUM MARIANUM*) ON SOME IMMUNE PARAMETERS OF RAINBOW TROUT, *ONCORHYNCHUS MYKISS* (ACTINOPTERYGII: SALMONIFORMES: SALMONIDAE)

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Background. Herbal medicines are increasingly used for their effects on the immune system. Silymarin, a mixture of flavonolignans from the seed of milk thistle (*Silybum marianum*), is used in treatment of liver disease, food and drug poising, and foetal diseases such as viral hepatitis, diabetes, and ischemia. Although, immunostimulant effects of the dietary silymarin were studied on different experimental animals, no information is available about the effects of silymarin on immune parameters of fish. The presently reported study was conducted to investigate the immunomodulatory effects of silymarin on some immunological and haematological parameters of rainbow trout.

Materials and methods. Juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792), were maintained in 1000 L fiberglass tanks at 15 ± 2ºC supplied with systems of water recirculation and aeration. Silymarin extract incorporated into diets (0.0, 0.1, 0.4, and 0.8 g of per 1 kg of feed) of fish. The trout were fed silymarin-supplemented diet for 30 days. Haematological parameters, such as red blood cell count (RBC), white blood cell count (WBC), haematocrit (Hct), haemoglobin (Hb), differential leukocyte- and immunological parameters such as peroxidase, lysozyme, and complement activities, total protein, albumin and globulin levels were measured on day 7, 15, and 30 days of silymarin treatment.

Results. The results indicated that oral administration of silymarin in fish, after 15 and 30 days of experimental periods, might enhance haematological and immunological parameters including lysozyme and complement activities, total protein and globulin levels, compared to the controls.

Conclusion. The results suggest that oral administration of silymarin may be useful to strengthen the immune system in rainbow trout.

Keywords: silymarin, rainbow trout, haematological parameters, immunological parameters

INTRODUCTION

In fish therapy efforts reported from many parts of the world, chemical drugs in aquaculture industry have been replaced by herbal medicine (Dügenci et al. 2003, Citarasu et al. 2006). The role of plant extracts in stimulating the fish immune system challenged with bacterial-, parasitic-, and fungal agents has been the subject of many studies (Sivaram et al. 2004, Vasudeva Rao and Chakrabarti 2005, Citarasu et al. 2006, Vasudeva Rao et al. 2006, Divyagneswari et al. 2007). However, the effect of herbal drugs on the health status of fish has been overlooked in most of these studies. Consequently, not much is known about usage of commercial herbal medicine in aquaculture. Therefore, investigating the effects of these compounds after administration seems a necessity.

Liver failures and diseases, food and drug poising and foetal diseases such as viral hepatitis (El-Kamary et al. 2009), diabetes (Soto et al. 2003), ischemia (Oliveira et al. 2001, Canbek et al. 2008), etc. in human and laboratory animals can traditionally be treated by one of the most important herbal medicines called milk thistle, *Silybum marianum* (Family: Asteraceae; known also as Compositae). The

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active constituents of *S. marianum* may strengthen or stimulate the immune response by interacting with various parameters of the immune system. The reported positive effect of herbal medicine has been expressed by a number of mechanisms, e.g., inhibition of tumor necrosis factor TNF-α, interferon IFN-γ, interleukin IL-2, IL-4, and nuclear factor-kappa B (NF-κB) activation in rat (Wilasrusmee et al. 2002, Ardestani and Yazdanparast 2007), inhibition of fibrosis in rat (Jia et al. 2001), inhibition of inflammation (Kaur and Agarwal 2007, Ramasamy and Agarwal 2008), immunomodulation in mice (Schümann et al. 2003), inhibition of mitochondrial injury in rat (Rolo et al. 2003), inhibition of P450 activity in human liver microsomes (Beckmann-Knopp et al. 2000), antioxidant properties and inhibition of lipid peroxidation in rat and fish (Han et al. 2007, Toklu et al. 2007, Banaee et al. 2011, Banaee unpublished[*]), enhancement of RNA, DNA (Sonnenbichler et al. 1984), protein synthesis in liver tissue of rainbow trout (Banaee et al. 2011), regulation of cell permeability (Kiruthiga et al. 2007, Basiglio et al. 2009), and adjustment of enzyme levels activity in plasma (Banaee et al. 2011).

Although the effect of oral administration of silymarin on blood biochemistry of fish has previously been studied (Banaee et al. 2011), no information is available on possible effect of silymarin, used as a feed supplement on different parameters of immune system of fish. Measuring the alterations in innate immunity and non-specific immune parameters of fish treated with herbal derivatives may be a good method to evaluate the effects of a herbal drug on immune system of fish. Therefore, the aim of the presently reported study was to assess the effect of oral administration of silymarin on some non-specific immune parameters in blood of rainbow trout.

MATERIALS AND METHODS

Fish and experimental procedure. Healthy rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792), average weight 90 ± 15 g were obtained from a private farm (Rainbow trout farm, Kordan village, Karaj, Iran.). They were maintained in closed water recirculating systems (1000 L) at the optimal laboratory conditions (temperature 15 ± 2ºC; pH: 7.4 ± 0.2; photoperiods: 16L : 8D) in the Biology Laboratory of Aquaculture Department, Tehran University. After two weeks of acclimation, the fish were randomly divided into four groups by triplicate that each contained 12 fish. Fish were fed commercial diets (Behparvar Co. Karaj, Iran) twice a day, equivalent to 2% of their body weight. During 30-day experiment, intended to examine the effect silymarin on immune parameters, the fish feed was supplemented with 0.1, 0.4, and 0.8 g silymarin extract per 1 kg of feed, while the controls were given the commercial diet only. The silymarin extract was prepared according to procedure described by Banaee et al. (2011). The formulated fish feed was prepared in the laboratory using powder of the commercial feed mentioned earlier. Finally, the 100, 400, and 800 mg of silymarin extracts as powder were mixed with 1 kg of commercial feed powder to achieve doses of 0.1, 0.4, and 0.8 g per kg of fish feed.

During the trials, the fish appetite was assessed based on the volume of the digestive system contents. Immunostimulatory activity was evaluated on day 7, 15, and 30 of the experimental periods; 12 fish per treatment were captured and anesthetized within aquatic solution of clove powder (as powder of dried flower) (1 : 5000). Fish from each group (experimental and control) were bled from the caudal vein into sterilized glass vials at 4°C containing the anticoagulant (1% EDTA). The blood was centrifuged for 15 min at 4000 × G, 4ºC. Plasma were immediately stored at –78ºC until biochemistry and immunostimulatory activity analysis.

Haematological parameters. The blood was immediately used to determine the number of red blood cells (RBC) and white blood cells (WBC) by means of a haemocytometer slide at a magnification of 400 ×. Subsequently, blood was diluted to 10^{-2} and 10^{-3} in phosphate buffered saline (PBS), at pH 7.2 (Sarder et al. 2001). Haematocrit (Hct) was determined by the microhaematocrit method described by Brown (1988). Haemoglobin (Hb) concentration was conducted by using the cyanohaemoglobin method (Azizoglu and Cengizler 1996). To differentiate blood cell type, blood smears from triplicate samples were prepared according to Banaee et al. (2008) and examined at a magnification of 400 ×.

Alternative complement activity. Alternative complement activity (ACH50) was evaluated following the procedure of Yano (1992) using rabbit red blood cells (RaRBC). Briefly, RaRBC were washed and adjusted to 2×10^8 cell · mL^{-1} in ethylene glycol tetra-acetic acid magnesium-gelatine veronal buffer (0.01 M). 100 µL of the RaRBC suspension was lysed with 3.4 mL of distilled water and the absorbance of the haemolysate was measured at 414 nm against distilled water to acquire the 100% lysis value. The test plasma was appropriately diluted, and different volumes ranging from 0.1 to 0.25 mL were made up to 0.25 mL total volume before being allowed to react with 0.1 mL of RaRBC in test tubes. After incubation at 20ºC for 90 min with occasional shaking, 3.15 mL of a 0.9% (v/v) saline solution was added to each tube with centrifugation at 1600 × G for 10 min at 4ºC. The absorbance (A) of supernatant was measured using a spectrophotometer at 414 nm. A lysis curve was obtained by plotting the percentage of haemolysis against the volume of plasma added. The volume of plasma producing 50% haemolysis (ACH50) was determined and the number of ACH50 units · mL^{-1} was obtained for each fish.

Lysozyme activity. The turbidimetric assay for lysozyme activity was carried out according to Lange et al. (2001) with minor modifications. Thus, plasma (50 µL) was added to 2 mL of a suspension of *Micrococcus lysodeikticus* (Actinobacteria: Micrococcaceae) (0.2 mg mL^{-1}) in a 0.05 M sodium phosphate buffer (pH 6.2). The reaction was carried out at 25ºC and absorbance was measured at 570 nm after 0.5 min and 4.5 min by spectrophotometer. PBS was used as the blank. Lysozyme of sample calibrated using a standard curve determined with hen's egg white lysozyme (Sigma) in PBS. The specific activity (units/ml plasma) for lysozyme was determined.

[*] Banaee M. 2010. Tasyer silymarin dar kahesh stress oxidative ijad shode nashi az samyat zir koshandhe diazinon dar mahi gezel alaey rangin Kaman (*Oncorhynchus mykiss*). [Influence of silymarin in decline of sub-lethal diazinon-induced oxidative stress in rainbow trout (*Oncorhynchus mykiss*)]. PhD Thesis; Aquaculture and Environmental Department, Natural Resource Faculty, Natural Resource and Agriculture Collage, Tehran University, Iran. [In Persian.]

Peroxidases content. The total peroxidase content present in plasma was measured according to Cuesta et al. (2007) with modification. Briefly, 10 µL of plasma was diluted with 100 µL of Hank's balanced salt solution (HBSS). Then, 50 µL of 20 mM 3,3′,5,5′–tetramethylbenzidine hydrochloride and 2.5 mM H_2O_2 were added. The colour change reaction was stopped after 2 min by adding 50 µL of 2 M sulphoric acid and the optical density (OD) was read at 450 nm. Standard samples without plasma were also analyzed. The peroxidase activity (units · mL^{-1} plasma) was determined defining one unit of peroxidase as that which produces an absorbance change of 1 OD.

Blood biochemical parameters. Plasma total protein and albumin levels were measured by using the total protein and albumin kit (Parsazma Co. Iran). Globulin levels were calculated by subtracting albumin values from plasma total protein.

Statistical analyses. Statistical analyses were performed using SPSS (Release 15) software. Data are presented as mean ± standard deviation. For all data, normal distribution was confirmed by the Kolmogorov–Smirnov test. Data were analyzed by one-way analysis of variance (ANOVA). Means were compared by Tukey's test and a $P < 0.05$ was considered statistically significant.

RESULTS

The effects of oral administration of 0.4 and 0.8 g of silymarin on RBC were statistically significant on day 30 ($P < 0.05$). Hb concentrations increased significantly when fish were treated with diets enriched with 0.8 g of silymarin on days 15 and 30 when compared to control ($P < 0.05$). Hb was significantly higher in fish which were fed with 0.4 g of silymarin supplementary food on day 15 ($P < 0.05$). Hct value of fish fed 0.1 g of silymarin supplement was significantly higher than its value in control group on day 30. There was no significant difference in haematological parameters between all of treatments and control groups on day 7 (Table 1).

Leukocyte counts significantly increased in fish fed 0.1 g of silymarin-enriched diets on day 15. Thrombocyte count was significantly higher in fish fed 0.8 and 0.1 g of silymarin ($P < 0.05$) as compared to controls on day 15 and 30, respectively. Nevertheless, no significant changes in neutrophils and monocytes were observed during the experiments (Table 2.).

No significant change in peroxidase activity in plasma of fish fed silymarin supplement was observed when compared with control group during experimental periods. ACH50 levels increased significantly when fish were treated with diets enriched by 0.4 g of silymarin during the experiments. Generally, lysozyme activity increased significantly in fish fed 0.1 and 0.4 g of silymarin on day 15 and 30 ($P < 0.05$) (Table 3).

Total protein levels significantly increased in the fish fed enriched by 0.1 g of silymarin on day 7, 15, and 30 of treatment when compared with control group ($P < 0.05$). In addition, a significant increase was observed in total protein plasma levels of treated fish by 0.4 and 0.8 g of silymarin on day15 and 30 ($P < 0.05$). Albumin levels in plasma of fish fed feed enriched by 0.1 g of silymarin per kg was significantly higher than control group on day 15 ($P < 0.05$). There was a significant elevation in the globulin levels in plasma of fish fed by food enriched with 0.1 g of silymarin during experimental periods ($P < 0.05$).

Table 1

Principal haematological parameters of *Oncorhynchus mykiss* fed diet containing silymarin

HP	Treatment [g · kg^{-1}]	Day of experiment		
		7	15	30
RBC [10^6 · µL^{-1}]	Control	1.14 ± 0.06^a	1.30 ± 0.13^{ab}	1.18 ± 0.12^a
	0.1	1.18 ± 0.08^a	1.22 ± 0.11^a	1.23 ± 0.10^{ab}
	0.4	1.21 ± 0.11^a	1.46 ± 0.16^b	1.40 ± 0.09^b
	0.8	1.16 ± 0.03^a	1.39 ± 0.15^{ab}	1.38 ± 0.13^b
WBC [10^4 · µL^{-1}]	Control	12.07 ± 0.31^a	12.38 ± 0.20^a	12.37 ± 0.94^a
	0.1	12.54 ± 0.53^a	12.92 ± 1.08^{ab}	14.29 ± 2.74^a
	0.4	12.79 ± 0.73^a	14.13 ± 1.70^b	13.41 ± 2.17^a
	0.8	12.28 ± 0.62^a	12.50 ± 0.51^{ab}	13.93 ± 1.36^a
Hb [g · dL^{-1}]	Control	10.10 ± 1.90^a	8.35 ± 1.63^a	8.78 ± 0.93^a
	0.1	11.24 ± 2.19^a	9.23 ± 1.21^{ab}	9.97 ± 2.17^{ab}
	0.4	11.17 ± 1.01^a	10.01 ± 0.41^{ab}	11.56 ± 1.19^b
	0.8	10.94 ± 1.01^a	10.74 ± 0.90^b	12.01 ± 0.92^b
Hct [%]	Control	39.32 ± 5.21^a	40.18 ± 2.56^a	38.88 ± 2.88^a
	0.1	42.83 ± 2.41^a	42.85 ± 1.76^a	43.65 ± 3.03^b
	0.4	38.63 ± 4.10^a	44.48 ± 4.01^a	40.85 ± 2.00^{ab}
	0.8	43.10 ± 1.97^a	43.98 ± 3.22^a	41.33 ± 1.86^{ab}

HP = haematological parameter; RBC = erythrocyte count; WBC = leukocyte count; Hct = haematocrit; Hb = haemoglobin; Treatment values express g of silymarin per 1 kg of feed; values not sharing identical superscript letters are significantly different (one-way ANOVA, $P < 0.05$; mean ± standard deviation).

Consumption of food containing 0.8 g of silymarin supplements had a significant effect on globulin concentrations in plasma of experimental fish on day 30 (Table 3).

DISCUSSION

The presently reported study investigated the immunostimulatory properties of silymarin—a milk thistle extract containing a large number of flavonolignans—including silybin, isosilybin, silydianin, silychristin, and dehydrosilybin, dehydrosilychristin, neosilyhermin, silyhermin, and silybinome (Banaee et al. 2011) on haematological- and immunological parameters of rainbow trout (*Oncorhynchus mykiss*). Recent studies have shown that herbal supplements to feed increased disease resistance in fish and improved survival and growth in rats, which may be attributed to improvement of immune functions (Christybapita et al. 2007, Divyagnaneswari et al. 2007, Ardó et al. 2008, Cheng et al. 2008).

Haematology, based on erythrocyte count, leukocyte count, haemoglobin concentration, and haematocrit has provided valuable information for fishery biologists in the assessment of fish health (Banaee et al. 2008). Our results suggest that oral administration of silymarin for at least 15 to 30 days may increase the number of erythrocytes and leukocytes as well as haematocrit and haemoglobin values. In other words, silymarin may effect the function of haematopoietic organs such as spleen and head kidney which play important role in blood cell formation. The results of this research revealed no significant alternations in differential leukocytes (lymphocytes, monocytes, and neutrophils) in the experimental fish when compared with control group. Increase in haemoglobin content (Hb), haematocrit, and numbers of leukocytes and thrombocyte were reported in Nile tilapia (Shalaby et al. 2006), hybrid tilapia (Ndong and Fall 2011) fed diet enriched by garlic. These results are in agreement with previous research in which feeding with other herbal supplemented feed led to an increase in erythrocyte- and leukocyte count, haemoglobin level and haematocrit (Martins et al. 2002, Ji et al. 2007).

Lysozymes are a family of enzymes with antibacterial activity characterized by the ability to damage the cell wall of bacteria. Thus, the significant increase in lysozyme activity in plasma of fish after 15 and 30 days of feeding with diets enriched by 0.1 and 0.4 g of silymarin may indicate an improvement of defence mechanisms against bacterial agents. Yet, feeding for 7 days did not reveal a significant difference in its activity relative to the controls. Ardó et al. (2008) reported an increase in lysozyme activities of Nile tilapia, *Oreochromis niloticus* (L.), fed for 7 days with Chinese herbs, *Astragalus membranaceus* (Fabaceae) and *Lonicera japonica* (Caprifoliaceae). Furthermore, according to other reports the use of *Astragalus radix* ((Fabaceae; see Yin et al. 2006), *Eclipta alba* (Asteraceae; see Christybapita et al. 2007), and *Ganoderma lucidum* (Agaricomycetes: Ganodermataceae; see Yin et al. 2009) incorporated into the fish diets, fed for 14 to 60 days, led to a significant increases in the lysozyme activity.

Complement includes over 20 different plasma proteins that are produced by a variety of cells including, hepatocytes, macrophages, and gut epithelial cells. Some

Table 2

Leukocyte- and thrombocyte counts in blood of *Oncorhynchus mykiss* fed diet containing silymarin

HP	Treatment [g · kg^{-1}]	Day of experiment		
		7	15	30
Lymphocytes	Control	84.99 ± 1.02^{a}	86.66 ± 1.02^{ab}	83.66 ± 1.01^{a}
	0.1	83.49 ± 1.01^{a}	88.83 ± 1.01^{b}	83.99 ± 1.02^{a}
	0.4	84.83 ± 1.01^{a}	86.80 ± 1.03^{ab}	84.82 ± 1.02^{a}
	0.8	86.13 ± 1.03^{a}	84.49 ± 1.02^{a}	85.16 ± 1.01^{a}
Monocytes	Control	7.11 ± 1.15^{a}	6.43 ± 1.18^{a}	7.63 ± 1.11^{a}
	0.1	7.65 ± 1.07^{a}	5.31 ± 1.10^{a}	6.77 ± 1.17^{a}
	0.4	6.63 ± 1.13^{a}	6.03 ± 1.26^{a}	6.95 ± 1.14^{a}
	0.8	6.78 ± 1.15^{a}	5.44 ± 1.18^{a}	7.61 ± 1.14^{a}
Neutrophils	Control	1.62 ± 1.73^{a}	1.59 ± 1.43^{a}	2.00 ± 1.00^{a}
	0.1	2.00 ± 1.00^{a}	1.41 ± 1.46^{a}	1.90 ± 1.76^{a}
	0.4	1.91 ± 1.43^{a}	1.74 ± 1.36^{a}	1.62 ± 1.73^{a}
	0.8	1.41 ± 1.46^{a}	2.14 ± 1.18^{a}	1.78 ± 1.33^{a}
Thrombocytes	Control	5.86 ± 1.28^{a}	5.01 ± 1.33^{a}	6.63 ± 1.13^{ab}
	0.1	6.80 ± 1.12^{a}	4.19 ± 1.33^{a}	6.91 ± 1.19^{b}
	0.4	6.46 ± 1.13^{a}	5.38 ± 1.27^{ab}	6.27 ± 1.17^{ab}
	0.8	5.28 ± 1.35^{a}	7.76 ± 1.17^{b}	5.25 ± 1.21^{a}

HP = haematological parameter; Treatment values express g of silymarin per 1 kg of feed; values not sharing identical superscript letters are significantly different (one-way ANOVA, $P < 0.05$; mean ± standard deviation).

complement proteins bind to immunoglobulins or to membrane components of cells. The complement system is an essential and effective part of the innate immune system. It can rapidly distinguish and opsonize bacteria for phagocytosis by specialized phagocytes or destroy them directly by membrane disorder (Rooijakkers and van Strijp 2007). Thus, the increase of the complement activity (ACH50) in plasma of fish may help to identify and eliminate bacteria agents by phagocytosis. The enhancement of complement activity (ACH50) in plasma of fish fed the feed enriched with 0.4 g of silymarin may indicate an improvement of the capabilities of the fish immune system during the experimental period. In line with the results of the present study, several authors have reported an increase in complement activity following administration of different immunostimulants such as herbal derivatives (Jian and Wu 2003, 2004, Christybapita et al. 2007), sodium alginate (Bagni et al. 2005, Cheng et al. 2008), and vitamins C and E (Ortuño et al. 1999, 2001).

Peroxidases are a large family of enzymes which play important role as natural antibacterial agent in animal immune system, e.g., myeloperoxidase (Clark and Klebanoff 1975). Although, oral administration of silymarin did not significantly affect peroxidase activity in plasma of fish when compared with control group, peroxidase activity in plasma of fish fed 0.1 g of silymarin was higher than in fish given 0.8 g of silymarin after 30 days of feeding. Christybapita et al. (2007) recorded an increase in myeloperoxidase activity in tilapia fed diets supplemented with different levels of aqueous extract of *Eclipta alba*, for 1 week, whereas they did not report any significant changes in myeloperoxidase activity after 2 or 3 weeks.

In the presently reported study, the potential enhancement of total protein by using silymarin-supplemented feed was investigated in fish. Banaee et al. (2011) reported that oral administration of silymarin might improve protein synthesis in fish liver tissue. Consequently, a significant increase of the total protein levels in plasma in

Table 3

Blood biochemical parameters of *Oncorhynchus mykiss* fed diet containing silymarin

BP	Treatment [g·kg^{-1}]	Day of experiment		
		7	15	30
Px [U·mL^{-1}]	Control	121.33 ± 7.00^{a}	115.83 ± 8.47^{a}	111.50 ± 7.82^{ab}
	0.1	124.00 ± 7.10^{a}	119.00 ± 10.02^{a}	124.00 ± 7.64^{b}
	0.4	116.67 ± 6.15^{a}	112.83 ± 7.57^{a}	117.33 ± 7.23^{ab}
	0.8	115.67 ± 7.47^{a}	110.50 ± 12.34^{a}	106.67 ± 11.99^{a}
Ac [U·mL^{-1}]	Control	311.17 ± 6.88^{a}	307.50 ± 9.50^{a}	311.67 ± 14.38^{a}
	0.1	326.17 ± 11.84^{ab}	323.50 ± 10.84^{ab}	330.50 ± 14.53^{ab}
	0.4	337.17 ± 13.01^{b}	340.50 ± 11.78^{b}	343.33 ± 17.51^{b}
	0.8	310.33 ± 10.78^{a}	316.00 ± 12.18^{a}	323.67 ± 7.66^{ab}
Ly [U·mL^{-1}]	Control	113.67 ± 5.54^{a}	117.00 ± 5.02^{ab}	112.17 ± 7.88^{a}
	0.1	118.83 ± 7.33^{a}	122.83 ± 6.49^{b}	125.00 ± 3.74^{b}
	0.4	122.33 ± 14.18^{a}	130.33 ± 12.63^{b}	135.17 ± 8.70^{b}
	0.8	111.83 ± 7.68^{a}	107.83 ± 9.62^{a}	102.83 ± 7.25^{a}
Pr [mg·dL^{-1}]	Control	3.95 ± 0.16^{a}	3.95 ± 0.16^{a}	4.08 ± 0.16^{a}
	0.1	4.87 ± 0.66^{b}	5.12 ± 0.92^{b}	4.65 ± 0.20^{b}
	0.4	4.75 ± 0.75^{ab}	4.23 ± 0.21^{b}	4.62 ± 0.34^{b}
	0.8	4.47 ± 0.41^{ab}	4.22 ± 0.19^{b}	4.72 ± 0.27^{b}
Al [mg·dL^{-1}]	Control	1.95 ± 0.16^{a}	1.92 ± 0.15^{a}	1.95 ± 0.08^{a}
	0.1	2.37 ± 0.33^{a}	2.43 ± 0.45^{b}	2.17 ± 0.26^{a}
	0.4	2.30 ± 0.36^{a}	2.12 ± 0.21^{ab}	2.17 ± 0.12^{a}
	0.8	2.23 ± 0.39^{a}	1.93 ± 0.08^{a}	2.12 ± 0.13^{a}
Gl [mg·dL^{-1}]	Control	2.00 ± 0.00^{a}	2.03 ± 0.08^{a}	2.13 ± 0.16^{a}
	0.1	2.50 ± 0.35^{b}	2.68 ± 0.49^{b}	2.48 ± 0.12^{b}
	0.4	2.45 ± 0.40^{ab}	2.12 ± 0.35^{a}	2.45 ± 0.29^{ab}
	0.8	2.23 ± 0.26^{ab}	2.28 ± 0.16^{ab}	2.60 ± 0.24^{b}

BP = Biochemical parameter; Px = peroxidase; Ac = ACH50; Ly = lysozyme; Pr = protein; Al = albumin; Gl = globulin; Treatment values express g of silymarin per 1 kg of feed; values not sharing identical superscript letters are significantly different (one-way ANOVA, $P < 0.05$; mean ± standard deviation).

treated fish is probably reflected increase of protein synthesis in liver tissue. Similarly, the highest serum protein level was recorded in Nile tilapia fed yellow leader and Japanese honeysuckle (Ardó et al. 2008), ginger, mistletoe, and stinging nettle (Dügenci et al. 2003). Proteins include albumin and globulin; some globulins are produced in the liver, while others are made by the immune system (Sandnes et al. 1988). Globulin is made up of subunit of α1, α2, β, and γ globulins, which are considered as the source of almost all the immunologically active proteins in the blood (Jha et al. 2007). Commonly, increases in the levels of plasma total protein, albumin and globulin in fish are thought to be associated with a stronger innate response (Wiegertjes et al. 1996). Although albumin did not increase in most of the treatment groups in the present study, globulin tended to respond similarly to total protein, which significantly increased in all experimental groups on day 15 and 30. Since albumin plays an important role in transport of some compounds such as drugs in blood, minor increase albumin levels in plasma of experimental fish may aid the transport of silymain in blood. Therefore, the increase of globulins in plasma of fish treated by silymarin may indicate an enhancement of immune system of fish.

In conclusion, the results indicated that the use the incorporation of silymarin as immunostimulants into fish feed might lead to enhanced health- and immune parameters in blood of the fish stimulated this way. Haematological studies recorded increases in RBC, Hct, and Hb following the administration of the silymarin-supplemented feed.

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