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Original Article

Effect of Milk thistle plant, *Vitis vinifera* extract on immune system of rainbow trout (*Oncorhynchus mykiss*) challenge by diazinon

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Abstract: The pollutants due to effect on the immune system of fish increase fish sensitivity to pathogens. Diazinon is one of the most used organophosphates pesticide in many agricultural areas. This study aimed to evaluate the effect of diazinon on the immune system of rainbow trout (*Oncorhynchus mykiss*) and application of Milk thistle plant, *Vitis vinifera* extract to reduce the adverse effects of this pesticide on its immune system. The reduction in the level of plasma peroxides, IgM, total complement and lysozyme were observed in fish exposed to diazinon showing its effect on the fish's immune system. No significant difference between control group and fish fed by Milk thistle plant extract and exposed to diazinon can reflect protective impact of Milk thistle plant extract on the immune system of rainbow trout by eliminating the free radicals and boosting the immune system.

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Introduction

The immune system of aquatic organisms such as fishes is continuously affected by unwanted environmental changes, threatening their health as an acute or chronic stressor. With overuse of pesticides, herbicides and other toxic compounds, the incidence of disorders and increase of irreparable damages are inevitable in aquatic organisms. Therefore, they can influence the immune system of the fishes. Diazinon is one of the most used agricultural organophosphate pesticides during past decade in Iran (Selsele, 2001; Tartahi Tbrizi, 2001; Honarpajo, 2003; Qomqisi, 2004; Hosseini, 2005; Babai, 2009). A significant reduction of white blood cells, particularly lymphocytes and increase in neutrophils and eosinophils were observed in *Acipenser stellatus* (Khoshbavar Rostami et al., 2005), *A. nudiventris* (Khoshbavar Rostami and Sultani, 2005), *Oncorhynchus mykiss* (Banaii et al., 2009) and *Cyprinus carpio* (Banaii et al., 2008) treated by

diazinon. In addition, a change in the activity level of lysozyme were reported in *Ctenopharyngodon idella* (Pourgholam and Sultani, 2007) and *O. mykiss* (Banaii et al., 2009) under treat of diazinon. Production of the free radicals by the diazinon's metabolism has been proven in various tissues, especially in the liver of the treated fishes (Üner et al., 2006) threatening their survival.

Animals' immune system has a relatively high sensitivity to oxidative stress. Glutathione (GSH) as the most important intracellular regenerative factor has various functions such as protection against oxidative stress, regulation of gene expression, regulation of apoptosis and activation and proliferation of T lymphocytes; however, it is observed that the low level of GSH is associated with the incidence of functional disorders in lymphocytes (Droge et al., 2000; Townsend et al., 2003). Therefore, strengthening of fishes' immune system by providing dietary supplements can be a suitable

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solution for preventing their immune system weakness. Many plants extracts directly reduce the effects of oxidative stress whereby the elimination of a variety of Reactive Oxygen Species (ROS) and can indirectly play their protective role through the activation of antioxidant defense system (Lang et al., 1988; Dietzmann et al., 2002; Townsend et al., 2003).

Medicinal plants as antifungal (Ebrahimzadeh Mousavi et al., 2006), antibacterial (Naghdi Badi and Makizadeh Tafti, 2007) and immunostimulant compounds (Ahmadi et al., 2008) have been used to increase the ability of fishes' immune system. Milk thistle, *Vitis vinifera* is considered as a cell protective compound, especially liver cells. Many studies have so far been conducted on the antioxidant properties, removing feature of oxy-radicals, the ability to chelate ion and addictive effects of intracellular glutathione content of *V. vinifera* showing its ability to enhance the antioxidant immune system (Borsari et al., 2001; Valenzuela and Garrido, 1994). However, the mechanism of Milk thistle plant extract has not been well-described as a stimulant of the immune system. Therefore, this study aims to evaluate the effect of Milk thistle plant extract on the immune system of *O. mykiss* in exposure to diazinon.

Materials and Methods

A total of 120 healthy rainbow trout (with mean weight of 85.5 ± 15 g) were randomly distributed into 12 100L-tanks with continuous aeration. Fish were adapted to in-vitro conditions for 15 days before experiments. During this period, fish were fed by a commercial trout grower diet (FFT2, Chineh, Tehran, Iran). For preparing experimental food, 400 mg Milk thistle plant extract per 1 kg of commercial food powder (FFT2, Chineh, Tehran, Iran) were mixed and the mixture was mixed with water to yield a suitable mash. The prepared diet was made into pellet and dried at 40°C in drying cabinet. Food was weekly prepared.

Toxicology test was performed according to OECD Guideline. During the experiment, the

physicochemical conditions of the water were regularly controlled. Chronic toxicity test was designed for 28 days as completely randomized design. Four treatment each with three replicates were considered for this study. In control group, fish only fed by commercial food; other treatments were fish fed by diet supplemented by Milk thistle plant extract, fish treated by diazinon (0.1 mg L^{-1}) and fish treated by diazinon (0.1 mg L^{-1}) and fed by diet supplemented by Milk thistle plant extract. The fish were reared in static water tanks with 100% water exchange on every other day. Throughout the experiment, the rearing water was constantly supplied with oxygen to maintain the dissolved oxygen above 6 mg L^{-1} . After each water change, the amount of pesticide was renewed.

The blood were randomly sampled from 3 fish of each tank at 7th, 14th and 28th days of the experiment. Plasma was separated after the centrifugation in 6000 rpm for 15 min at 4°C, then the samples were kept in -78°C for further analysis. To assess the level of peroxidase activity, 15 ml of plasma with 35 ml HBSS buffer without magnesium and calcium was diluted. Then 50 ml TMB solution and 5 mM hydrogen peroxide were added so that the solution turned blue. After 2 min, by adding 50 ml of 2 M sulfuric acid, color reaction was stopped and the color became bright yellow. The amount of optical absorption at 450 nm was measured to determine the peroxidase activity.

Measuring complement of CH50 was done by kit (Bahar Afshan Company, Tehran) based on Radial Immuno Diffusion method. Lysozyme activity level was measured by turbidity method using *Micrococcus lysodeikticus* suspension and Moramydaz enzyme. The turbidity was measured at a wavelength of 670 nm. IgM levels in plasma were measured using inkjet Tehran spring kit and Hitachi auto analyzer.

Statistical analysis was performed using Minitab software version 13 and diagrams were drawn by Microsoft Excel 2003. Statistical analysis was performed by one-way ANOVA and the significance of means was performed at 95% level of significance

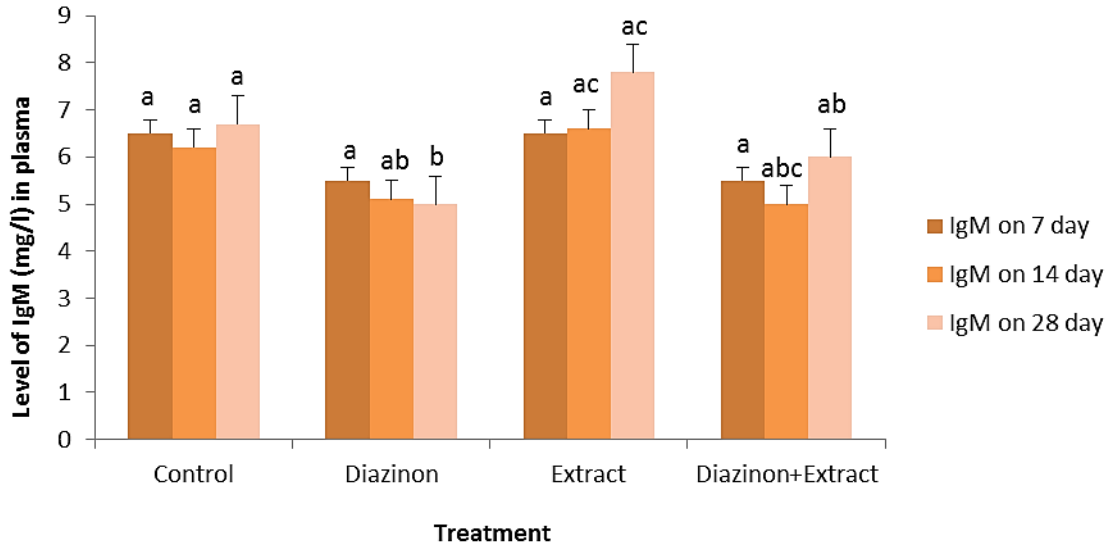


Figure 1. Change in the level of plasma immunoglobulin in *Oncorhynchus mykiss* treated by pesticide, Milk thistle plant extract and complement of Milk thistle plant extract and pesticide.

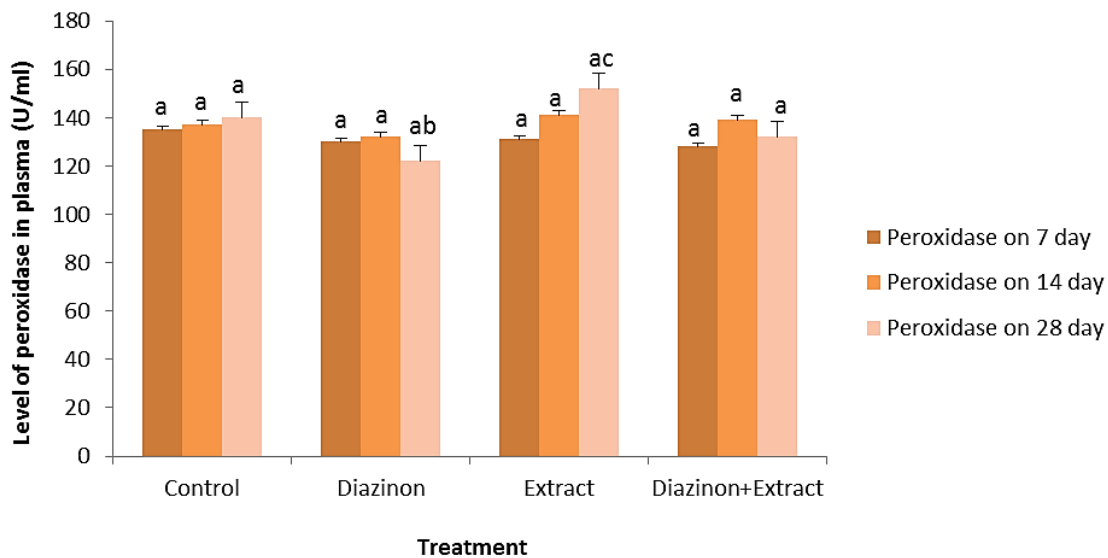


Figure 2. Change in the level of peroxidase in *Oncorhynchus mykiss* treated by pesticide, Milk thistle plant extract and complement of Milk thistle plant extract and pesticide.

applying Tukey test.

Results

During the experiment, no death were observed; however, fish treated by pesticide seemed to be nervous at the end of the trial period. Some fish were swimming as unbalanced in the water surface which was due to the deterrent effect of diazinon. Such behavior was not observed in other treatments. Changes in the level of immunoglobulin IgM and peroxidase, total complement and lysozyme were

shown Figures 1-4. At 14th and 28th days, the level of immunoglobulin was significantly decreased in fish treated by diazinon ($P < 0.05$); however, at 14th, no significant change was observed in the concentration of IgM in the treated fish.

No significant difference ($P < 0.05$) was observed in the level of peroxidase in the treated fish compared to the control group in all stages of sampling; whereas, a significant difference was observed between the level of peroxidase in fish treated by diazinon and fish fed with diet

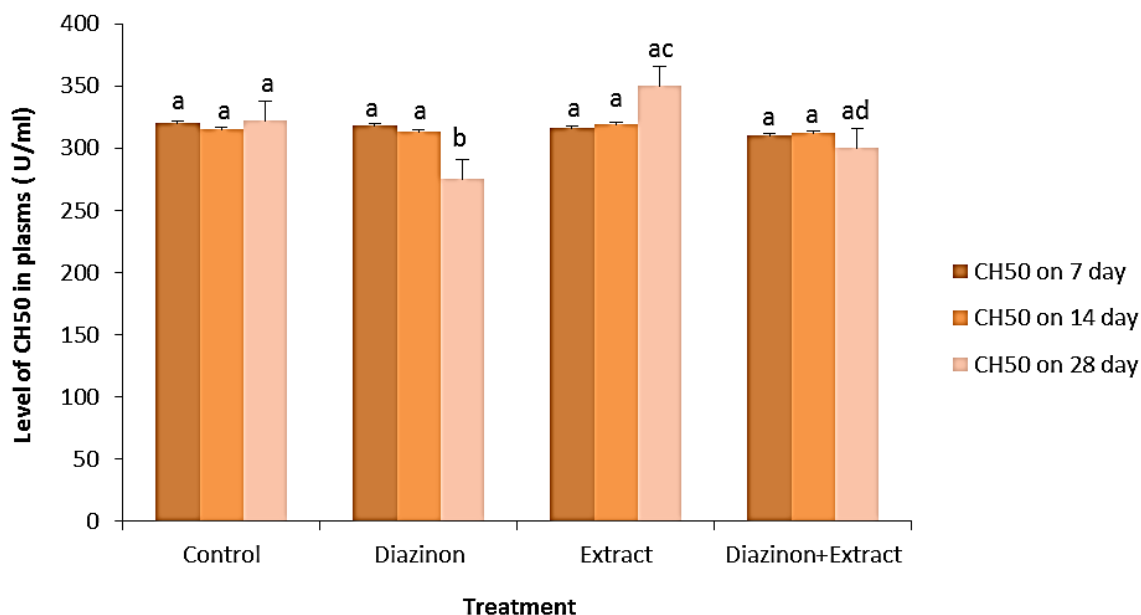


Figure 3. Change in the level of total complement in *Oncorhynchus mykiss* treated by pesticide, Milk thistle plant extract and complement of Milk thistle plant extract and pesticide.

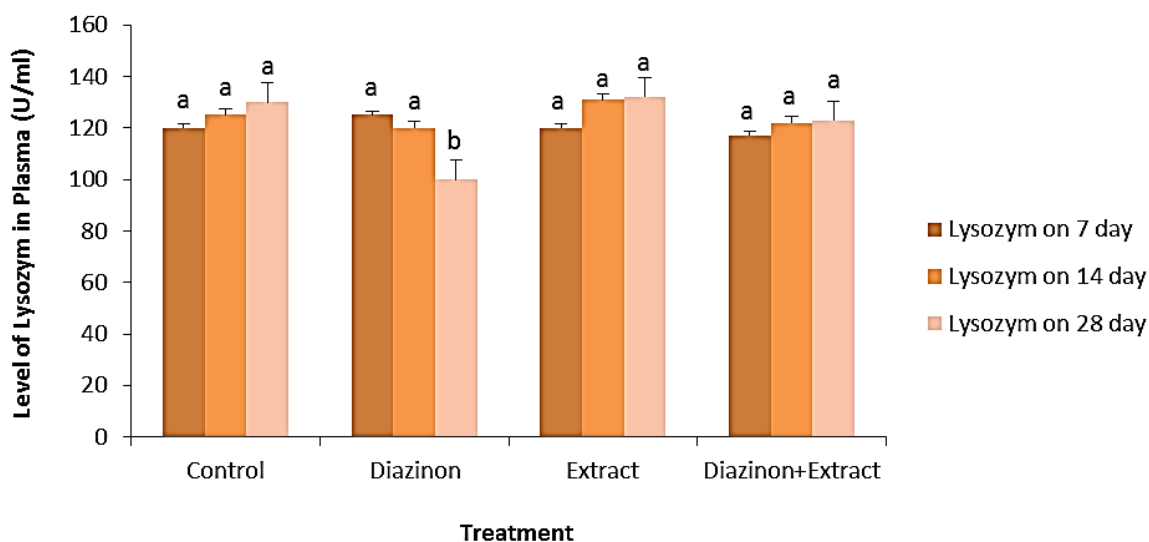


Figure 4. Change in the level of plasma lysosyme in *Oncorhynchus mykiss* treated by pesticide, Milk thistle plant extract and complement of Milk thistle plant extract and pesticide.

supplemented by Milk thistle plant extract at the third phase of the sampling ($P < 0.05$).

The level of total complements of plasma was significantly decreased in fish exposed to diazinon compared to control group after 28 days ($P < 0.05$). A significant increase ($P < 0.05$) in the level of plasma complements was also observed in fish fed by Milk thistle plant extract.

The level of lysozyme significantly decreased at the third phase of sampling in fish treated by pesticide compared to that of control group

($P < 0.05$); while in the other treatments, no significant differences were observed in the level of plasma lysozyme.

Discussion

In recent years, numerous studies have been conducted on the therapeutic effects of Milk thistle plant extract on various diseases and significant results have been achieved. In vitro studies have shown the therapeutic effects of plant extracts with similar combinations to Milk thistle plant on

hyperlipidemia, atherosclerosis and formation of atherosclerotic plaque (Kreman et al., 1998), poisoning and kidney disorders (Zima et al., 1998), drug toxicity (Muriel and Mourelle, 1990), kidney dysfunction (Fiebrich and Koch, 1979), food (Desplaces et al., 1975) and chemical poisoning (Janiak, 1974), viral diseases (Lirussi and Okolicsanyi, 1992), neurological disorders (Zhang et al., 1993), regulatory of blood sugar (Velussi et al., 1993), anti-cancer property (Zi et al., 1998) and prevention of hemolysis of red (Zou et al., 2001) and white blood cells (Locher et al., 1998).

Peroxide enzymes play significant role in oxidation of many xenobiotics by hydrogen peroxide (Saunders, 1973). In this study, the ability of antioxidant enzyme system to remove the free radicals produced through diazinon metabolism was increased in accordance with increasing the level of peroxidases' activity in the plasma of fish fed by complement of Milk thistle plant extract. While reducing the level of this enzyme in fish exposed to diazinon led to imbalance between the antioxidant defense system and production of ROS causing serious damages of their immune system. Free radicals produced during the metabolism and decomposition of diazinon in the detoxification process in the liver of fishes can cause disorder in the normal function through oxidation of intracellular proteins. The integrity and stability of membrane is eliminated due to the lipid peroxidation of the cellular membranes. It causes disorder in the cell interactions which will underlie cell death (Üner et al., 2006). Because the membrane of the immune cells is rich by long-chain polyunsaturated fatty acids, they are more susceptible to lipid peroxidation by ROS produced in active phagocytic cells (Üner et al., 2006).

Immunoglobulins or antibodies a class of glycoproteins are found in serum and tissues fluids of all vertebrates. Antibodies are produced by plasmocytes derived from B lymphocytes (Stites, 1991). Immunoglobulins play an important role in fight against bacterial infectious diseases. Therefore, reducing the level of their activity can weaken the

immune system of the fishes. In the present study, a significant reduction of immunoglobulin level indicates the effect of diazinon in reducing the level of plasma immunoglobulin in fish treated by diazinon. No significant difference among fish fed by Milk thistle plant extract and exposed to pesticide also reveals the effect of Milk thistle plant extract on reducing complications caused by diazinon.

According to the results, reducing the levels of total plasma complements in treatments exposed to diazinon can represent the effect of pesticide in weakening the fish's immune system, while Milk thistle plant extract can reduce the adverse impact of the free radicals on fish's immune system by the metabolism of diazinon through preventing cell death and increasing the regenerative capacity of tissues. Lymphocytes capability for proliferative response and production of IL-2 are deeply damaged in exposure to ROS (Flescher et al., 1994).

Decreasing the level of lysozyme in fish exposed to pesticide also clearly shows the impact of diazinon on weakening the immune system. Also, no significant difference between lysozyme of fish in the control group and those fed by Milk thistle plant extract can indicate the effect of Milk thistle plant extract on boosting the immune system (Pourgholam and Soltani, 2007).

As results, the extract of Milk thistle plant, particularly antioxidant compounds can prevent harmful effects of diazinon on the immune system of fish by eliminating the free radicals and boosting the immune system (Ditzmann et al., 2002; Levin et al., 1996; Heyborn and Silver, 1996). According to the results, the impact of Milk thistle plant extract as a medicinal extract was proved on improvement of the immune system. Therefore, using Milk thistle plant extract in fishes' diet can be a suitable solution to prevent of possible adverse effects of entering toxic pollutants into fish farms and their effects on fishes' immune system.

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