

Protective Effects of Silymarin Extract on Malathion-Induced Zebra Cichlid (*Cichlasoma Nigrofasciatum*) Hepatotoxicity

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ABSTRACT

Background: There is much evidence indicating that natural substances from edible and medicinal plants possess powerful antioxidant and hepatoprotective activities. The objective of the present study was to investigate the potential hepatoprotective effect of silymarin in fish exposed to malathion.

Methods: Zebra cichlid fish were allocated into five groups of which one group received normal feed and served as control. Fish from group 2 were treated with 0.1 mg.L⁻¹ malathion. Fish from group 3 and 4 were fed with enriched diet with 1400 mg and 2100 mg silymarin per 1 kg feed, respectively. While fish from group 5 and 6 were fed with enriched diet with 1400 mg and 2100 mg silymarin per 1 kg feed, respectively and simultaneously were treated with 0.1 mg.L⁻¹ malathion for 15 days. Activities of hepatic enzymes including alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and lactate dehydrogenase were evaluated. Oxidative stress was ascertained by measuring malondialdehyde as marker of lipid peroxidation and total cellular antioxidant capacity.

Results: Exposure to malathion caused a significant increase in MDA levels and altered AST, ALT, ALP and LDH activities in liver tissues ($p < 0.05$). The hepatic antioxidant capacity was significantly lowered in malathion treated fish as compared to the control group ($p < 0.05$). Treatment with silymarin significantly ameliorated these changes in the malathion-treated groups.

Conclusion: These finding demonstrated that silymarin have protective effects against the toxic influence of malathion on the examined biochemical parameters in liver tissue of fish.

Keywords: Hepatic Enzymes, Hepatoprotective, Malathion, Oxidative Stress, Silymarin.

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INTRODUCTION

There are many pathways by which pesticides are distributed throughout the aquatic ecosystems. The major route of pesticides contamination of surface waters is through rainfall runoffs and air-drift [1-3]. Aquatic organisms living in aquatic ecosystems close to agricultural fields are the most important non-target organisms that can be affected by pesticides. In surface waters pesticides may be absorbed through the gills, skin and digestive system of aquatic animals and distributed in different tissues through the blood. Due to the lipophilic property of pesticides, they accumulate mainly in fatty tissues. Bioaccumulation and detoxification of pesticides

in the liver may affect the physiological function of cells [4- 7].

Malathion, an organophosphate (OP) insecticide, is widely used in agriculture for various food and feed crops, homeowner outdoor uses, ornamental nursery stock, building perimeters, pastures and rangelands, and regional pest eradication programs [8]. Malathion is a potent environment toxicant which induces oxidative stress damage through production of highly reactive free radicals. Malathion is activated by cytochrome P450 system to form reactive radical like malaaxon which can react with cellular components. Reactive oxygen species (ROS) initiate lipid peroxidation, altering the phospholipid membranes and affecting their cellular

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permeability and finally leading to serve cell damage [9, 10]. The common protecting strategies of natural antioxidants include free radical scavenging ability and enhancing the endogenous antioxidant enzyme systems [11]. Plants naturally contain phenolic compounds and flavonoids with antioxidant properties capable of eliminating free radicals [12, 13]. Silymarin extracted from milk thistle, *Silybum marianum*, is a polyphenolic compound with anti-hepatotoxic properties [14]. This compound has been used worldwide over 200 years as a medical plant in the treatment of liver diseases and since the identification of this plant's clinical effects in 1969, its usage has increased [14, 15]. Banaee et al. [16] reported that silymarin extracts added to fish diet increased protein synthesis in liver tissue. Silymarin administration also modulates plasma enzyme activities, activated antioxidant enzymes and protects liver from the histopathological damage induced by diazinon [15, 16]. Ahmadi et al. [17] suggested that oral administration of silymarin may be useful to improve the immune parameters in fish.

The aim of the present study was to assess the hepatoprotective and antioxidant efficacy of silymarin extract against malathion-induced hepatotoxicity in a fish model.

MATERIALS AND METHODS

Chemicals

Malathion [O, O-dimethyl dithiophosphate of diethyl mercaptosuccinate] was purchased from Moshkfam Fars Company, Iran. Silymarin extract was obtained from GOLDARU Company, Iran.

The concentration of malathion and the doses of silymarin were selected according to an earlier study conducted in our laboratory. The preliminary acute toxicity test were performed in accordance with OECD guidelines to estimate the lethal concentration that cause 50% mortality at 96 h (96-h LC₅₀). The fish were exposed to different nominal concentrations of malathion at 27±2 °C in a static-renewal system, where water and pesticide were completely replaced every 24 h in 40 liter glass aquaria. The 96 h LC₅₀ value of malathion in Zebra cichlid (*C.nigrofasciatum*) was 1.14±0.13 mg.L⁻¹. For sub-lethal toxicity tests, the concentrations of malathion in water were maintained modestly below the 96 h LC₅₀

value. Based on this value, the sub-lethal concentration (10% of 96 h LC₅₀) was chosen for the Zebra cichlid (*C.nigrofasciatum*).

The concentration of silymarin for the current study was determined based on its antioxidant properties. Antioxidant activity of silymarin was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay [18]. Seven concentrations of silymarin were tested (175, 350, 700, 1400, 2100, 2800 and 3500 µg.mL⁻¹) which showed an increased antioxidant response of 13.20%, 31.60%, 55.20%, 86.60%, 97.00%, 86.30% and 54.60% inhibition, respectively. According with these results together with taking into account the IC₅₀ value for silymarin (513 µg.mL⁻¹), concentrations of 1400 and 2100 mg per kg of food were selected (Banaee, unpublished data).

Fish Treatments

Zebra cichlids (*Cichlasoma nigrofasciatum*) weighing 6.50 ± 0.75 g were used in the present study according to National Ethical Framework for Animal Research in Iran [19]. The specimens were housed in a number of 12 per aquaria at 27±2 °C under a daily photoperiod of 16 h light/ 8 h dark and were fed with pellet diet (BioMar Co.). Fishes were randomly assigned to six groups. Group I: specimens were fed with normal diet for 15 days, and were considered the control group. Group II: specimens were exposed to 0.1 mg.L⁻¹ malathion. Group III: specimens were fed with enriched diet with 1400 mg silymarin per 1 kg feed for 15 days. Group IV: specimens were fed with enriched diet with 2100 mg silymarin per 1 kg feed for 15 days. Group V and VI: specimens were exposed to 0.1 mg.L⁻¹ malathion and were fed with enriched diet with 1400 mg and 2100 mg silymarin per 1 kg feed for 15 days, respectively.

At the end of the experiment, specimens were euthanized by decapitation and livers were carefully removed, washed repeatedly in ice-cold physiological saline and accurately weighed. Tissue samples were homogenized for two minutes in ice cold phosphate buffer (pH 7.4; 1:10, w/v) using a glass homogenizer and then were centrifuged for 15 min at 15000 g at 4°C in a refrigerated centrifuge. Supernatants were immediately used to measure biochemical parameters by using spectrophotometric assays.

Biochemical Analysis

Aspartate aminotransferase (AST) was assayed in a coupled reaction with malate dehydrogenase in the presence of NADH [20]. In the alanine aminotransferase (ALT) assay, the enzyme reacts with alanine and α -ketoglutarate to form glutamate and pyruvate [20]. Lactate dehydrogenase (LDH) activity determination was based on measuring the conversion of pyruvate to L-lactate by monitoring the NADH oxidation. LDH converts pyruvate to lactate and NAD^+ [20]. All these activities were monitored by measuring the change in absorbance at 340 nm. Alkaline phosphatase (ALP) assay was based on enzyme-mediated conversion of *p*-nitrophenol phosphate to nitrophenol in an alkaline buffer at 405 nm [20]. Levels of protein in liver tissue were determined by standard procedure used in clinical biochemistry laboratories based on manual biochemical kits (ParsAzemon Co, Iran) [21].

Total antioxidant capacity was assayed according to the ferric reducing ability of plasma (FRAP) method. Briefly, the FRAP reagent contained 5 mL of a (10 mmol/L) TPTZ (2,4,6-tripyridyl-*s*-triazine) solution in 40 mmol/L HCL plus 5 mL of FeCl_3 (20 mmol/L) and 50 mL of acetate buffer, (0.3 mol/L, pH=3.6) and was prepared freshly. Aliquots of 100 μL supernatant were mixed with 3 mL FRAP reagent. The conversion rate of ferric tripyridyl-*s*-triazine (Fe^{3+} -TPTZ) complex to ferrous tripyridyl-*s*-triazine (Fe^{2+} -TPTZ) at pH 3.6 and temperature 25° C is directly proportional to the concentration of total antioxidant in the sample. Fe^{2+} -TPTZ has an intensive blue color that can be monitored for up to 5 min at 593 nm by a UV/VIS spectrophotometer. Calculations were performed using a calibration curve of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (100 to 1000 $\mu\text{M/L}$) [22].

Malondialdehyde (MDA) content was assessed by a modification of a thiobarbituric acid assay and was expressed as $\mu\text{mol/g}$ tissue [23]. Briefly, 500 μl of the supernatant was transferred to a Pyrex tube and mixed with 2500 μl trichloroacetic acid (20%) and 1000 μL trichlorothiobarbituric acid (67%). The tubes were then placed in boiling water (100°C) for 15 min. After cooling, the mixture was extracted with a solution containing 1000 μL of distilled water and 5000 μL *n*-butanol: pyridine (15: 1). The

mixture was then centrifuged at 2000 g for 15 min at 4° C. The pink color produced by these reactions was measured spectrophotometrically at 532 nm to measure MDA levels. MDA concentration was calculated with an external standard of MDA. Tetraethoxypropane and absolute ethanol were used to prepare the MDA standards. Concentrations of MDA in whole body samples are expressed in μM per g protein.

All biochemical parameters were measured in duplicate in a UV/Vis Unico spectrophotometer (model 2100) at room temperature (25 °C)

Statistical Analysis

All the data were examined for normality (Kolmogorov-Smirnov test). Statistical tests were performed with SPSS (IBM, Release 19) software by means of one way analysis of variance, followed by Tukey multiple comparison test ($p < 0.05$). Data are presented as mean \pm SD in each experimental group. Significant differences between values were characterized by alphabetical symbols ($p < 0.05$).

RESULTS

No mortality was observed during the experiment in any of the aquaria. Increased mucus secretion, color changes and behavioral changes such as tremors, lethargy, unbalanced swimming, swimming in the surface water and extreme irritability were important alterations evidenced in the individuals exposed to malathion.

The activity of aspartate aminotransferase (AST) was significantly increased in the livers of malathion treated fish ($p < 0.05$). Fish fed with diets enriched by silymarin extract had no significant change in the AST activity compared with control values ($p > 0.05$). AST activity was not significantly reduced in fish simultaneously exposed to malathion and supplemented with silymarin (Figure 1).

The activity of alanine aminotransferase (ALT) was significantly increased in the liver of malathion treated fish ($p < 0.05$). The supplementation with silymarin extract maintained the activity of ALT to similar to the control group (Figure 2).

The activity of alkaline phosphatase (ALP) in the livers of fish, fed with enriched diet with 2100 $\text{mg} \cdot \text{Kg}^{-1}$ silymarin was significantly lower

than ALP activity in the livers of fish exposed to malathion ($p < 0.05$).

The activity of lactate dehydrogenase (LDH) was significantly increased in the livers of malathion treated fish ($p < 0.05$). Groups treated with silymarin had similar activities as the control group.

The levels of total antioxidant were significantly decreased in the liver cells of fish

exposed to malathion ($p < 0.05$). Administration of silymarin extract in fish exposed to malathion had no significant effect on the cellular total antioxidant capacity.

The levels of malondialdehyde (MDA) were significantly increased in the livers of fish exposed to malathion ($p < 0.05$). Administration of silymarin ameliorated lipid peroxidation but not reaching the control values.

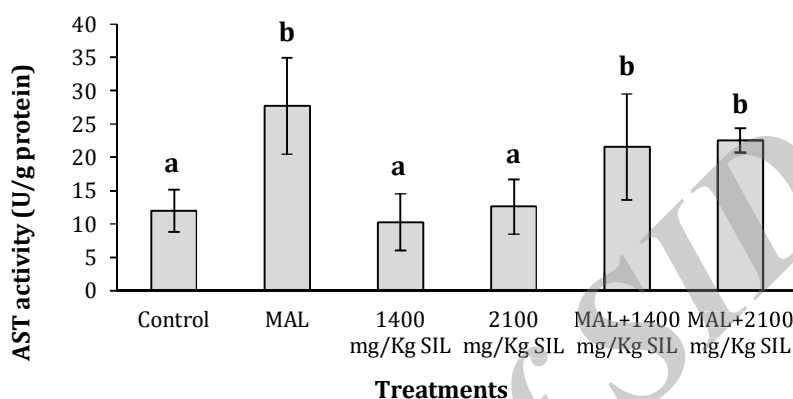


Figure 1. Effect of silymarin on the liver AST activity of the control and experimental fish.

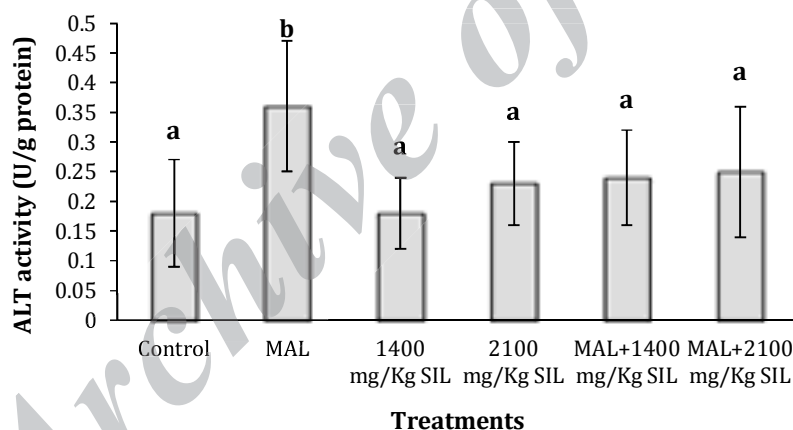


Figure 2. Effect of silymarin on the liver ALT activity of the control and experimental fish.

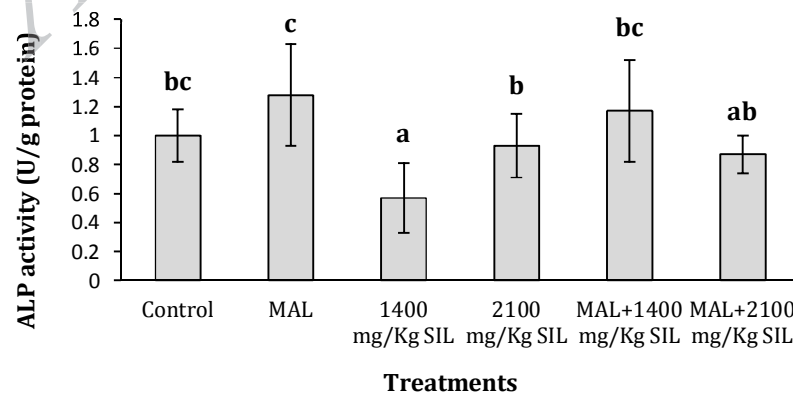


Figure 3. Effect of silymarin on the liver ALP activity of the control and experimental fish.

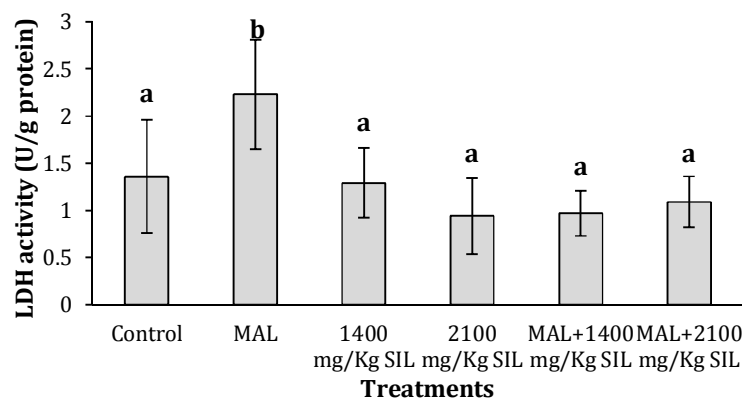


Figure 4. Effect of silymarin on the liver LDH activity of the control and experimental fish.

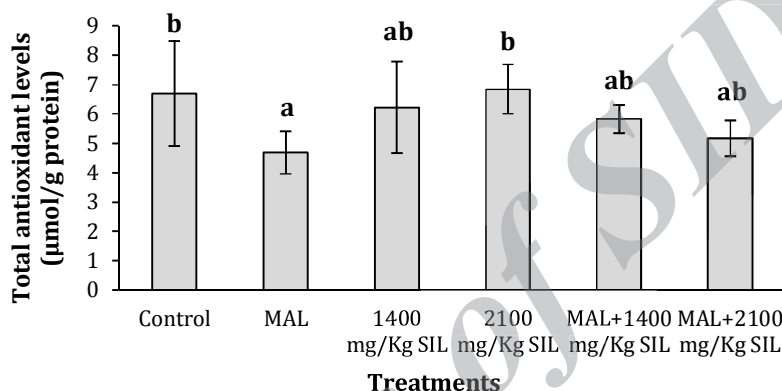


Figure 5. Effect of silymarin on the cellular total antioxidant levels of the control and experimental fish.

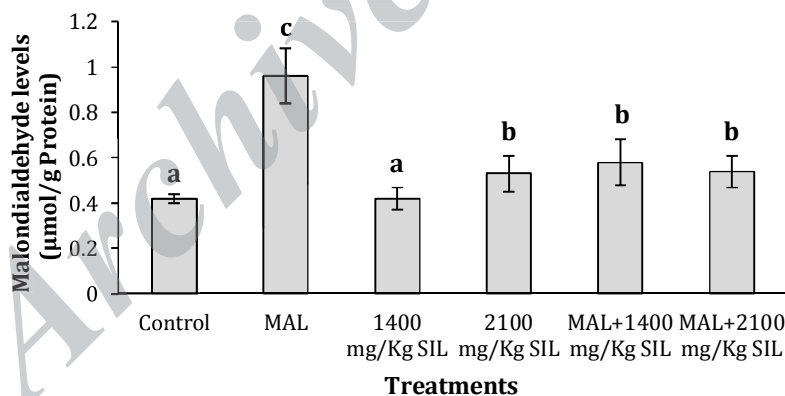


Figure 6. Effect of silymarin on the malondialdehyde levels of the control and experimental fish.

DISCUSSION

Several studies have recently been carried out to investigate the possible protective effect of various natural substances having antioxidant properties on xenobiotic-induced tissue damage associated with lipid peroxidation [24- 28]. Silymarin is an antihepatotoxic polyphenolic substance isolated from the milk thistle plant, *Silybum marianum* [14]. The hepatoprotective properties of silymarin have been confirmed in

various experimental animals exposed to different xenobiotics [29- 31]. Moreover, there is a lack of investigation about the influence of silymarin on malathion toxicity. This study was designed to investigate whether silymarin could reduce the oxidative stress in the liver tissue of fish exposed to malathion.

Although, no mortality was recorded during the experimental period for all treatment groups, some of the specimens exposed to

malathion exhibited signs of toxicity. Increased mucus secretion, color changes, behavioral changes, lethargy, unbalanced swimming, swimming in surface water and extreme irritability were important changes observed in some fish exposed to malathion during experimental periods. These changes were intensified at the end of experiment, supporting the toxic effects of malathion.

Malathion is a well-known organophosphate pesticide, capable of inducing oxidative stress by generating potent reactive oxygen substances as a result of its biotransformation. These ROSs react with the cellular macromolecules and induce lipid peroxidation. This leads to the loss of cellular membrane integrity resulting in pores in the cell membranes. AST, ALT, ALP and LDH are enzymes usually present in the liver cells, when hepatic tissue is damaged these enzymes leak out from the cells into blood leading to increased levels and activities in plasma [2]. Our results showed malathion-induced changes in the activities of various enzymes such as AST, ALT, ALP and LDH in liver tissue of fish. These alterations in hepatic enzymes are biomarkers of hepatic injury and indicated severe hepatic damage caused by malathion. These results are corroborated by previous reports evaluating pesticides induced toxicity in various fish [6, 32-34]. The present data showed that oral administration of silymarin extract to fish exposed to malathion maintains the activity of ALT, ALP and LDH in similar values as the control group. In accordance, previous studies showed that oral administration of silymarin can regulate AST, ALT, ALP and LDH activities in plasma of laboratory mice which were experimentally poisoned with various drugs [15, 31].

In the present study, increased lipid peroxidation due to malathion exposure was accompanied by a depletion of hepatic total antioxidant capacity in the livers of exposed fish. Similar results were observed in *Ooncorhynchus mykiss* and *Alburnus mossulensis* after exposure to diazinon and fenprothrin [3, 6]. The overproduction of free radicals during pesticide detoxification may be associated with a decrease in the hepatic total antioxidant capacity [6, 35]. Therefore, the increase in the MDA levels in the present study could be related to an increase in

the generation of ROS in the liver of fish exposed to malathion. Increases in MDA levels were reported in different tissues of fish exposed to other pesticides such as fenprothrin [6], deltamethrin [36], methyl parathion and chlorpyrifos [37], carbamazepine [38], and trazine [39].

Cellular total antioxidant capacity is critical to ensure physiological functions and protection against free radicals. In our experiments the oral administration of silymarin did not influence the hepatic total antioxidant levels in fish exposed to malathion. Nonetheless, our results demonstrated a significant effect of silymarin treatment as a hepatoprotective agent in oxidative stress which significantly decreases lipid peroxidation, expressed as MDA levels, in liver tissue of fish exposed to malathion. Flavonoids and vitamin E in the silymarin extract are well known to be highly efficient radical scavengers [15, 40-42]. Thus, this decrease in MDA may be due to the free radical scavenging properties of silymarin. Therefore, simultaneous administration of silymarin to the pesticide exposure restricted the lipid peroxidation by inhibiting the metabolism of malathion and production of free radicals in liver cells.

CONCLUSION

In summary, the results from the present research showed that malathion induced oxidative damage in liver tissue with increased MDA levels. The toxicity of malathion leads to changes in the normal cellular enzyme activities, and a reduction in the cellular total antioxidant capacity. Diet supplementation with silymarin extract resulted in protective effects against the toxic influence of malathion on some examined biochemical parameters in the liver tissue of fish. Our findings demonstrated that silymarin may be helpful in reducing the adverse effects of malathion by maintaining optimum cellular biochemical hemostasis. However, further investigations are necessary to elucidate the mechanism of silymarin mediated protection against pesticide toxicity in aquatic animals.

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REFERENCE

1. Banaee M, Mirvaghefi A, Rafei G, Majazi Amiri B. Effect of sub-lethal diazinon concentrations on blood plasma biochemistry. 2008;2(2): 189-198.
2. Banaee M, Sureda A, Mirvaghefi A, Ahmadi K. Effects of diazinon on biochemical parameters of blood in rainbow trout (*Oncorhynchus mykiss*). Pesticide Biochemistry and Physiology. 2011;99(1):1-6.
3. Banaee M, Sureda A, Mirvaghefi AR, Ahmadi K. Biochemical and histological changes in the liver tissue of Rainbow trout (*Oncorhynchus mykiss*) exposed to sub-lethal concentrations of diazinon. Fish physiology and biochemistry. 2013;39(3):489-501.
4. Banaee M, Sureda A, Mirvaghefi A, Ahmadi K. Histopathological Alterations Induced by Diazinon in Rainbow trout (*Oncorhynchus mykiss*). Int J Environ Res. 2013;7(3):735-44.
5. Banaee M, Haghi BN, Ibrahim ATA. Sub-lethal toxicity of chlorpyrifos on Common carp, *Cyprinus carpio* (Linnaeus, 1758): Biochemical response. International Journal of Aquatic Biology. 2014;1(6):281-8.
6. Banaee M, Sureda A, Zohiery F, NematdoustHagi B, Garanzini DS. Alterations in biochemical parameters of the freshwater fish, *Alburnus mossulensis*, exposed to sub-lethal concentrations of Fenpropathrin. International Journal of Aquatic Biology. 2014. Article in Press.
7. Ahmadi K, Mirvaghefi AR, Banaee M, Vosoghei AR. Effects of long-term diazinon exposure on some immunological and haematological parameters in rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792). Toxicology and Environmental Health Sciences. 2014;6(1):1-7.
8. Bondarenko S, Gan J, Haver DL, Kabashima JN. Persistence of selected organophosphate and carbamate insecticides in waters from a coastal watershed. Environmental Toxicology and Chemistry. 2004;23(11):2649-54.
9. Chen X-y, Shao J-z, Xiang L-x, Liu X-m. Involvement of apoptosis in malathion-induced cytotoxicity in a grass carp (*Ctenopharyngodon idellus*) cell line. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 2006;142(1):36-45.
10. Possamai F, Fortunato J, Feier G, Agostinho F, Quevedo J, Wilhelm Filho D, et al. Oxidative stress after acute and sub-chronic malathion intoxication in Wistar rats. Environmental toxicology and pharmacology. 2007;23(2):198-204.
11. Kaur G, Alam MS, Jabbar Z, Javed K, Athar M. Evaluation of antioxidant activity of *Cassia siamea* flowers. Journal of ethnopharmacology. 2006;108(3):340-8.
12. Montvale NJ. PDR for herbal medicines: Medical Economics Company. 2000. p. 516-20.
13. Huang B, Ban X, He J, Zeng H, Zhang P, Wang Y. Hepatoprotective and antioxidant effects of the methanolic extract from *Halenia elliptica*. Journal of ethnopharmacology. 2010;131(2):276-81.
14. Kvasnička F, Břiba B, Ševčík R, Voldřich M, Kratka J. Analysis of the active components of silymarin. Journal of Chromatography A. 2003;990(1):239-45.
15. Banaee M. Influence of silymarin in decline of sublethal 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, 2010. [Persian]
16. Banaee M, Sureda A, Mirvaghefi AR, Rafei GR. Effects of long-term silymarin oral supplementation on the blood biochemical profile of rainbow trout (*Oncorhynchus mykiss*). Fish physiology and biochemistry. 2011;37(4):885-96.
17. Ahmadi K, Banaee M, Vosoghei AR, Mirvaghefi AR, Ataeimehr B. Evaluation of the immunomodulatory effects of silymarin extract (*Silybum marianum*) on some immune parameters of rainbow trout, *Oncorhynchus mykiss* (Actinopterygii: Salmoniformes: Salmonidae). Acta Ichthyologica Et Piscatoria. 2012;42(2):113-20.
18. Iwashima M, Mori J, Ting X, Matsunaga T, Hayashi K, Shinoda D, et al. Antioxidant and antiviral activities of plastoquinones from the brown alga *Sargassum micracanthum*, and a new chromene derivative converted from the plastoquinones. Biological and Pharmaceutical Bulletin. 2005;28(2):374-7.
19. Mobasher M, Aramesh K, Aldavoud S, Ashrafganjooei N, Divsalar K, Phillips C, et al. Proposing a national ethical framework for animal research in Iran. Iranian Journal of Public Health. 2008;37(1 Sup):39-46.
20. Moss DV, Henderson AR. Clinical enzymology In: Burtis CA, Ashwood ER, editors. Tietz Textbook of Clinical Chemistry. 3rd ed. Philadelphia: W.B. Saunders Company. 1999. 617-721.
21. Johnson AM, Rohlfs EM, Silverman LM. Proteins. In: Burtis CA, Ashwood ER, editors. Tietz Textbook of Clinical Chemistry. 3rd ed.

- Philadelphia: W.B. Saunders Company. 1999; p. 477-540.
22. Benzie IF, Strain J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical biochemistry*. 1996;239(1):70-6.
 23. Placer ZA, Cushman LL, Johnson BC. Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems. *Analytical biochemistry*. 1966;16(2):359-64.
 24. Yonar SM. Toxic effects of malathion in carp, *Cyprinus carpio*: Protective role of lycopene. *Ecotoxicology and Environmental Safety*. 2013; 97: 223-9.
 25. Krithika R, Verma RJ. Ameliorative effects of phyllanthin on carbon tetrachloride-induced hepatic oxidative damage in mice. *Asian Pacific Journal of Tropical Disease*. 2014;4:S64-S70.
 26. Raj S, Gothandam K. Hepatoprotective effect of polyphenols rich methanolic extract of *Amorphophallus commutatus* var. *wayanadensis* against CCl₄ induced hepatic injury in swiss albino mice. *Food and Chemical Toxicology*. 2014;67:105-12.
 27. Shaaban AA, Shaker ME, Zalata KR, El-Kashef HA, Ibrahim TM. Modulation of carbon tetrachloride-induced hepatic oxidative stress, injury and fibrosis by olmesartan and omega-3. *Chemico-biological interactions*. 2014;207:81-91.
 28. Wang D, Zhao Y, Sun Y, Yang X. Protective effects of Ziyang tea polysaccharides on CCl₄-induced oxidative liver damage in mice. *Food Chemistry*. 2014; 143: 371-8.
 29. Asgari S, Madani H, Naderi GA, TooriSh, TalebAlhosseini M. Hepatoprotective Effect of *Silybummarinum* (L.) Gaerten, and *Glycyrrhizglabral* in The Rats. *Journal of Medicinal Plants*. 2005; 4:18-24.[Persian]
 30. Madani S, Taleb Alhosseini M, Asgary S, Naderi G. Hepatoprotective effects of silybum marianum and *calendula officinalis* polyphenolic extracts in rat. *Iranian Journal of Biology*. 2006;19(2):157-63.
 31. Shaarawy SM, Tohamy AA, Elgendy SM, Elmageed ZYA, Bahnasy A, Mohamed MS, et al. Protective effects of garlic and silymarin on NDEA-induced rats hepatotoxicity. *International journal of biological sciences*. 2009;5(6):549.
 32. Velisek J, Svobodova Z, Machova J. Effects of bifenthrin on some haematological, biochemical and histopathological parameters of common carp (*Cyprinus carpio* L.). *Fish physiology and biochemistry*. 2009;35(4):583-90.
 33. Tripathi G, Shasmal J. Reparation of chlorpyrifos-induced impairment by thyroxine and vitamin C in fish. *Ecotoxicology and environmental safety*. 2010;73(6):1397-401.
 34. Banaee M, Ahmadi K. Sub-lethal toxicity impacts of endosulfan on some biochemical parameters of the freshwater crayfish (*Astacus leptodactylus*). *Research Journal of Environmental Sciences*. 2011;5(11):827-35.
 35. Monteiro DA, De Almeida JA, Rantin FT, Kalinin AL. Oxidative stress biomarkers in the freshwater characid fish, *Brycon cephalus*, exposed to organophosphorus insecticide Folisuper 600 (methyl parathion). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*. 2006;143(2):141-9.
 36. Yonar ME, Sakin F. Ameliorative effect of lycopene on antioxidant status in *Cyprinus carpio* during pyrethroid deltamethrin exposure. *Pesticide Biochemistry and Physiology*. 2011;99(3):226-31.
 37. Sharbidre AA, Metkari V, Patode P. Effect of methyl parathion and chlorpyrifos on certain biomarkers in various tissues of guppy fish, *Poecilia reticulata*. *Pesticide Biochemistry and Physiology*. 2011;101(2):132-41.
 38. Li Z-H, Velisek J, Zlabek V, Grabic R, Machova J, Kolarova J, et al. Hepatic antioxidant status and hematological parameters in rainbow trout, *Oncorhynchus mykiss*, after chronic exposure to carbamazepine. *Chemico-biological interactions*. 2010;183(1):98-104.
 39. Paulino M, Sakuragui M, Fernandes M. Effects of atrazine on the gill cells and ionic balance in a neotropical fish, *Prochilodus lineatus*. *Chemosphere*. 2012;86(1):1-7.
 40. Zuber R, Modrianský M, Dvořák Z, Rohovský P, Ulrichová J, Šimánek V, et al. Effect of silybin and its congeners on human liver microsomal cytochrome P450 activities. *Phytotherapy Research*. 2002;16(7):632-8.
 41. Shalan M, Mostafa M, Hassouna M, El-Nabi SH, El-Refaie A. Amelioration of lead toxicity on rat liver with vitamin C and silymarin supplements. *Toxicology*. 2005;206(1):1-15.
 42. Doehmer J, Tewes B, Klein K-U, Gritzko K, Muschick H, Mengs U. Assessment of drug-drug interaction for silymarin. *Toxicology in Vitro*. 2008;22(3):610-7.