Journal of ENVIRONMENTAL Treatment Techniques

J. Environ. Treat. Tech.
ISSN: 2309-1185
Journal web link: http://www.jett.dormaj.com

Effects of Sub-lethal Concentrations of Atrazine on Some Oxidative Stress Biomarker in Various Tissues of Grass Carp (*Ctenopharyngodon idella*)

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Received: 25/12/2014 Accepted: 25/01/2015 Published: 30/03/2015

Abstract

In the present study, activity of catalase (CAT), total antioxidant (TA), glutathione (GSH) and malondialdehyde (MDA) content in gills, liver and kidney tissues were measured after the grass carp (*Ctenopharyngodon idella*) exposure to 3, 6 and 15 mg/L atrazine for 10, 20 and 30 days. A significant increase in TA levels was observed in kidney of fish exposed to atrazine during experimental periods. TA in liver of fish exposed to 15 mg/L atrazine were significantly higher than control group on day 20 and 30. However, CAT activity (on day 10 and 20) and GSH levels (on day 30) increased in liver and kidney of fish exposed to 15 mg/L atrazine, no significant changes were observed in CAT activity and GSH levels in gills of fish. Although there were no significant changes in MDA levels in liver and kidney during experimental periods, MDA levels significantly increased in gills of fish exposed to 15 mg/L atrazine on day 30. Increased antioxidant capacity and decreased oxidative stress risk in grass carp after exposure to atrazine may be related to the fish diet. Our results show that fish fed with alfalfa may increase the efficiency of the antioxidant defense system against oxidative stress.

Keywords: Atrazine, oxidative stress, antioxidant status, grass carp, alfalfa

1 Introduction

Khuzestan Province is one of the most important agricultural centers in Iran [1], because a significant proportion of Iran's freshwater resources are located in this province. The application of various pesticides and chemical fertilizers in Khuzestan Province is much higher than international standards. Therefore, without any doubt it can be claimed that the province's surface water and groundwater is contaminated with different pesticides [2, 3, 4]. However, there is little information about the contamination of surface water and groundwater with various pesticides in Khuzestan Province. Contamination of surface waters and underground waters in this province is a serious threat to residents' health and aquatic ecosystems.

Atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-1,3,5-triazine) is the most widely used agricultural herbicide in Khuzestan Province, Iran. Although atrazine is relatively persistent in freshwater, its half-life in fresh water depending on physio-chemical conditions of the water varies from 8 to 350 days [5, 6]. Since Khuzestan Rivers are important habitats for freshwater fishes in Iran, pollution of river water with this herbicide may have negative effects on the health and survival of fish. Reproductive disorders including histopathological changes in testis and sex steroids and alterations in hepatic metabolism and oxidative stress were reported in fish after exposure to atrazine [6, 7, 8, 9, 10].

Like other pesticides, atrazine may be absorbed through the gills, skin and digestive system in fish. After this herbicide enters the fish body, atrazine is degraded by cytochrome P450 in the Phase I of detoxification, and its metabolites are excreted from body after conjugation with glutathione in the Phase II of detoxification [10]. So, decreased cellular glutathione levels and increased active metabolites of atrazine during detoxification process may cause oxidative stress in different tissues of fish.

Grass carp (*Ctenopharyngodon idella*) is one of the most important cultured fish in Iran. Also grass carp and other species belonging to the family Cyprinidae are found in most rivers in Iran, so it is an appropriate model species to study the toxicity of pesticides. Therefore, the aim of this study is to identify the changes found in some biomarkers of oxidative stress in different tissues of grass

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carp exposed to sub-lethal concentrations of commercial formulation of atrazine.

2 Materials and Methods

2.1 Experimental animals

Healthy grass carp (*Ctenopharyngodon idella*) (body weight: 50 ± 10 g), were purchased from a local fish farm and acclimated to the laboratory conditions for 2 weeks before the experiments. Fishes were randomly parcelled in 12 closed 1000-L recirculating tanks supplied with oxygenated water maintaining constant dissolved oxygen at 6.5 ± 0.5 mg/L, temperature at 24.5 ± 2 °C, pH at 7.4 ± 0.2, water hardness 250 ± 5 mg/L $CaCO_3$ and natural photoperiod. During acclimation and all experimental tests, fishes were fed with fresh alfalfa. Fishes were deprived of food for 24 h before sacrifice.

2.2 Acute toxicity experiments

Two hundred and forty immature grass carp (*Ctenopharyngodon idella*) were used in acute toxicity tests. The acute toxicity test was conducted following the OECD Guideline No. 203 under static-renewal test conditions. Test solutions of atrazine were prepared from a commercial atrazine [6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine], WP 80%, were purchased from Moshkfam Fars Co. Iran. Nominal concentrations of atrazine were selected from 0 (control), 10, 100, 500, 750 and 1500 mg/L. Thus, the acute toxicity was conducted with six different concentrations of pesticides and four replicates for each concentration in 24 aquariums (100 L). During the 96 h acute toxicity experiment, water in each fiberglass tank was aerated and had the same conditions as the acclimation period. The static-renewal tests exposed the fish for 96 h with replacement of the test solution every 24 h (all stock solutions were made immediately prior to use). The water was changed daily to reduce the build-up of metabolic wastes and to keep concentrations of atrazine near the nominal level. Fish mortality was recorded 0, 24, 48, 72, and 96 h after exposure to atrazine. LC_{50} values were calculated by the Probit Analysis test [11].

2.3 Sub-lethal toxicity experiments

One hundred and twenty grass carp were randomly distributed in nine 100 L aquariums (3 treatments by triplicate) to perform the 30 day period sub-lethal toxicity tests. Every tank containing 10 fishes were exposed to test solutions with the following concentrations of atrazine: 0.0 (control), 3 (5% 96h LC_{50}), 6 (10% 96h LC_{50}) and 15 mg/L (25% 96h LC_{50}), respectively. Sub-lethal concentrations were selected according to the previous acute toxicity test. The water was changed daily to reduce the build-up of metabolic wastes and to keep concentrations of atrazine near the nominal level. Nine fishes per each exposure concentration were captured and anesthetized with extract of clove powder (200 mg/L) after 10, 20 and 30 days of exposure. Fishes were sacrificed and the gills, liver and kidny tissue samples were collected and washed by 0.6% saline solution. The samples were immediately stored at -78°C until biochemical analysis.

Gill, liver and kidney samples were homogenized during two minutes in ice-cold phosphate buffer (pH 7.4; 1:10, w/v) using a glass homogenizer and then centrifuged for 15 min at 15000 g at 4°C with a refrigerated centrifuge. Supernatants were immediately used to determine total antioxidant capacity, reduced glutathione, malondialdehyde, catalase and total protein content by using a spectrophotometric assay.

2.4 Biochemical Analysis

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 up to 5 min at 593 nm by a UV/VIS spectrophotometer. Calculations were performed using a calibration curve of $FeSO_4 \cdot 7H_2O$ (100 to 1000 µM/L) [12].

Reduced glutathione was measured following the method of Beutler *et al.* [13] with some modifications. 50 µL of supernatant was added to 2700 µL of buffer phosphate solution (pH: 8) and 50 µL (5, 5´-dithiobis-2-nitrobenzoic acid (DTNB) was then added to this solution. GSH reacts with DTNB which produces a yellow colored compound, 5 'thio-2-nitrobenzoic-acid (TNB). The rate of TNB production is directly proportional to this recycling reaction which is in turn directly proportional to the concentration of GSH in the sample. Concentration of TNB was measured at 412 nm wavelength.

Malondialdehyde (MDA) content was assessed by a modification of a thiobarbituric acid assay and was expressed as µmol/g tissue [14]. 500 µl of the supernatant was transferred to a Pyrex tube and mixed with 2500 µl tricholoroacetic acid (20%) and 1000 µmL triclorthiobarbituric acid (67%). The tubes were then placed in boiling water (100°C) for 15 min. After cooling, the mixtures were 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 and compared with an external standard of MDA. Tetraethoxypropane and absolute ethanol were used to prepare the MDA standards.

CAT activity was determined according to Góth [15] method with some modifications. Catalase activity was measured by an assay of hydrogen peroxidase based on formation of its stable complex with ammonium molybdate. 50 µL of supernatant was incubated in working solution including 1000 µL hydrogen peroxide and 500 µL phosphate buffer (pH: 7.4) at 25 °C for 10 min. Then 1000 µL ammonium molybdate was added to the reaction solution and the concentration of the yellow complex of molybdate and hydrogen peroxide was measured at 405 nm wavelengths.

The biochemical parameters mentioned above were expressed per mg protein in liver tissue determined by the Biuret method using bovine serum albumin as a standard.

2.5 Statistical analysis

Statistical analyses were performed using SPSS (Release 19) software. Data are presented as mean ± SD.

All the data were tested for normality (Kolmogorov-Smirnov test). Data were analyzed by one-way of variance analysis (ANOVA). The significant means were compared by Duncan's test and a $p < 0.05$ was considered statistically significant.

3 Results

Numerical value of LC_{50} of atrazine at 24, 48, 72 and 96 hours are presented in Table 1. Fish mortality elevated with increasing the concentration of atrazine.

Sub-lethal concentrations of atrazine were determined according to 96h LC_{50} (equal to 5, 10 and 25% of 96h LC_{50}. No mortality was observed in fish exposed to sub-lethal concentrations of atrazine and control group during experimental periods. Alterations in oxidative stress biomarkers in gills tissue of fish after exposure to atrazine are presented in Table 2.

Table 1: Numerical value of lethal concentrations at different times

Lethal Concentrations	Numerical value of lethal concentrations at different times (mg/L)			
	24 hours	48 hours	72 hours	96 hours
LC99	1163.43±106.46	593.61±65.55	553.51±70.20	194.25±26.60
LC90	818.14±69.37	416.69±44.56	380.23±46.79	133.96±17.25
LC80	672.75±55.24	342.19±36.42	307.26±37.60	108.57±13.60
LC70	567.91±46.30	288.47±31.10	254.65±31.50	90.26±11.21
LC60	478.34±40.11	242.57±27.14	209.70±26.92	74.622±9.46
LC50	394.61±36.18	199.67±24.19	167.68±23.48	60.00±8.25
LC40	310.88±34.68	156.77±22.27	125.66±21.26	45.38±7.64
LC30	221.30±36.10	110.87±21.64	80.71±20.673	29.740±7.81
LC20	116.46±41.27	57.15±22.93	28.10±22.51	11.44±9.01

Table 2: Alterations in oxidative stress biomarkers in gills tissue of fish after exposure to atrazine

Biochemical Parameters	Concentrations of Atrazine	Periods when the fish were exposed to atrazine		
		10^{th} day	20^{th} day	30^{th} day
Total antioxidant (mM/g)	0.00 mg/L	79.47±12.05^{ab}	86.83±25.26^{a}	90.90±19.06^{a}
	3.00 mg/L	65.74±29.07^{a}	76.86±17.55^{a}	90.88±20.75^{a}
	6.00 mg/L	87.37±32.93^{ab}	164.59±63.05^{b}	113.12±50.03^{a}
	15.00 mg/L	100.69±22.17^{b}	237.29±7.56^{c}	183.04±89.41^{b}
GSH (µg/g)	0.00 mg/L	3828.54±965.30^{a}	3477.15±451.24^{a}	3919.49±944.73^{a}
	3.00 mg/L	3338.10±1342.63^{a}	3652.38±1204.17^{a}	4319.05±181.30^{a}
	6.00 mg/L	4442.86±1923.30^{a}	3519.05±1014.61^{a}	4095.24±698.02^{a}
	15.00 mg/L	4776.19±1652.52^{a}	4813.14±2060.44^{a}	4455.38±647.27^{a}
MDA (µM/g)	0.00 mg/L	1.21±0.28^{a}	1.21±0.06^{a}	1.21±0.06^{a}
	3.00 mg/L	1.12±0.35^{a}	1.01±0.05^{a}	1.09±0.28^{a}
	6.00 mg/L	1.49±0.34^{a}	1.09±0.22^{a}	1.42±0.46^{ab}
	15.00 mg/L	1.18±0.42^{a}	1.16±0.61^{a}	1.62±0.25^{b}
CAT (µM/g)	0.00 mg/L	38.11±14.41^{a}	33.11±10.58^{a}	37.09±16.14^{a}
	3.00 mg/L	35.68±27.13^{a}	32.47±14.06^{a}	32.10±34.54^{a}
	6.00 mg/L	49.17±30.31^{a}	30.63±16.29^{a}	34.56±18.97^{a}
	15.00 mg/L	50.32±14.91^{a}	41.16±13.81^{a}	32.05±14.58^{a}

Although, no significant changes were observed in total antioxidant levels in gill tissue of fish on day 10 of the experiment, total antioxidant levels significantly increased in gill tissue after fish exposure to 6 and 15 mg/L atrazine on day 20 of the test. Total antioxidant levels in gill tissue of fish exposed to 15 mg/L atrazine were significantly higher than control group on day 30 of the trial. Results show that there were no significant changes in GSH levels in gill tissue of fish during experimental periods. Although there were no significant changes in MDA levels in gill tissue of fish on day 10 and 20 of the test, MDA levels significantly increased in gill tissue of fish exposed to 15 mg/L atrazine on day 30 of the trial. No significant changes were observed in CAT activity in gill tissue of fish exposed to atrazine when compared with control group. Changes in oxidative stress biomarker in liver tissue of fish exposed to atrazine are presented in Table 3.

Total antioxidant levels in liver tissue of fish exposed to 15 mg/L atrazine were significantly higher than control group on day 10 and 30 of the trial. A significant increase in total antioxidant levels was observed in fish after exposure to 6 and 15 mg/L atrazine on day 20 of the test. Although no significant changes were observed in GSH levels in liver tissue of fish on day 10 of the experiment, GSH levels in liver tissue of fish exposed to 15 mg/L atrazine were significantly higher than control group on day 20 of the trial. GSH levels in liver tissue significantly increased after fish exposure to 6 and 15 mg/L atrazine on day 30 of the test. There were no significant changes in MDA levels in liver tissue of fish during experimental periods. However, CAT activity levels increased in liver tissue of fish after exposure to 15 mg/L atrazine on day 10 and 20 of the trial, there were no significant changes in CAT activity levels in liver tissue of fish on day 30 of the test.

Alterations in oxidative stress biomarkers in kidney tissue of fish after exposure to atrazine are presented in Table 4. Total antioxidant levels significantly increased in fish exposed to atrazine during experimental periods. GSH levels in kidney tissue of fish exposed to 15 mg/L atrazine were higher than control group on day 10 of the experiment. Although no significant changes were observed in GHS levels in kidney tissue of fish on day 20 of the experiment, GSH levels significantly increased in kidney tissue after fish exposure to atrazine on day 30 of the test. There were no significant changes in MDA levels in Kidney. CAT activity levels significantly increased in kidney tissue of fish exposed to 15 mg/L atrazine on day 10 and 20 of the trial, however no significant changes were observed in CAT activity in fish on day 30 of the experiment.

4 Discussion

Physiological response of fish exposed to pesticides to oxidative stress depends on the fish species, gender,

living conditions, nutrition, etc. Although several studies have been done on oxidative stress caused by Atrazine in various fish [8], physiological response of any fish was different when compared with other species.

Table 3: Alterations in oxidative stress biomarkers in Liver tissue of fish after exposure to atrazine

Biochemical Parameters	Concentrations of Atrazine	Periods when the fish were exposed to atrazine		
		10^{th} day	20^{th} day	30^{th} day
Total antioxidant (mM/g)	0.00 mg/L	94.69±66.15^{a}	101.34±66.15^{a}	102.21±42.37^{a}
	3.00 mg/L	157.62±25.03^{ab}	177.36±64.42^{ab}	128.91±47.63^{a}
	6.00 mg/L	168.50±48.87^{ab}	238.64±36.87^{bc}	221.70±96.45^{ab}
	15.00 mg/L	247.56±143.90^{b}	267.26±101.47^{c}	154.94±26.73^{b}
GSH (µg/g)	0.00 mg/L	7859.29±1251.22^{a}	7594.29±1251.22^{a}	6480.94±1585.70^{a}
	3.00 mg/L	9095.24±1393.24^{a}	8463.10±1323.15^{a}	6157.14±144.00^{a}
	6.00 mg/L	9300.09±2025.38^{a}	8990.48±1635.34^{a}	11671.43±686.19^{b}
	15.00 mg/L	9247.62±1262.22^{a}	11420.73±1017.41^{b}	9310.07±4209.93^{b}
MDA (µM/g)	0.00 mg/L	0.76±0.20^{a}	0.74±0.20^{a}	0.88±0.40^{a}
	3.00 mg/L	0.94±0.49^{a}	0.96±0.54^{a}	0.69±0.25^{a}
	6.00 mg/L	0.66±0.23^{a}	0.62±0.22^{a}	0.62±0.43^{a}
	15.00 mg/L	0.69±0.49^{a}	0.81±0.46^{a}	0.97±0.25^{a}
CAT (µM/g)	0.00 mg/L	93.35±3.69^{a}	91.34±3.69^{a}	104.79±13.94^{a}
	3.00 mg/L	120.85±32.57^{ab}	93.63±31.34^{a}	127.35±2.32^{a}
	6.00 mg/L	104.77±5.13^{ab}	128.49±5.91^{a}	113.37±55.61^{a}
	15.00 mg/L	160.10±85.06^{b}	196.45±55.42^{b}	136.33±44.62^{a}

Table 4: Alterations in oxidative stress biomarkers in kidney tissue of fish after exposure to atrazine

Biochemical Parameters	Concentrations of Atrazine	Periods when the fish were exposed to atrazine		
		10^{th} day	20^{th} day	30^{th} day
Total antioxidant (mM/g)	0.00 mg/L	84.38±24.23^{a}	74.72±31.84^{a}	79.72±42.23^{a}
	3.00 mg/L	167.28±40.62^{b}	159.89±41.39^{b}	173.88±28.46^{b}
	6.00 mg/L	169.97±55.71^{b}	299.68±67.68^{c}	161.41±59.00^{b}
	15.00 mg/L	137.48±21.81^{b}	257.26±43.27^{bc}	166.62±29.06^{b}
GSH (µg/g)	0.00 mg/L	5742.14±466.35^{a}	5985.71±403.86^{a}	5985.71±403.86^{a}
	3.00 mg/L	6980.95±1996.95^{ab}	6141.27±477.34^{a}	5552.38±652.70^{a}
	6.00 mg/L	7314.29±211.12^{ab}	7749.78±3303.91^{a}	7761.90±1092.11^{b}
	15.00 mg/L	8414.29±2517.17$_{b}$	8952.38±3076.10^{a}	10164.84±1145.35^{c}
MDA (µM/g)	0.00 mg/L	1.98±0.69^{a}	2.30±0.39^{a}	2.04±0.07^{a}
	3.00 mg/L	2.57±0.81^{a}	2.39±0.68^{a}	1.73±0.64^{a}
	6.00 mg/L	2.14±0.88^{a}	1.84±0.44^{a}	2.11±0.20^{a}
	15.00 mg/L	1.80±0.12^{a}	2.10±1.58^{a}	2.16±0.58^{a}
CAT (µM/g)	0.00 mg/L	98.46±11.02^{a}	101.38±1.35^{a}	102.82±11.93^{a}
	3.00 mg/L	103.70±14.77^{a}	102.51±4.13^{a}	121.11±4.84^{a}
	6.00 mg/L	110.57±19.10^{a}	116.83±7.62^{a}	126.13±47.27^{a}
	15.00 mg/L	222.60±77.67^{b}	203.39±46.88^{b}	155.04±67.41^{a}

Increasing antioxidant levels may be a defense mechanism to neutralize free radicals generated during the detoxification process of atrazine in different tissues of grass carp. Although, glutathione conjugated with metabolites of atrazine in the phase II of detoxification, no significant changes were observed in GSH levels in gill tissue of fish exposed to atrazine during experimental periods. A significant increase in cellular glutathione levels in liver and kidney tissues of fish exposed to atrazine may indicate an increased regeneration rate of GSH from oxidized glutathione (GSSG). Reinforcement of antioxidant system in liver and kidney tissues of fish exposed to atrazine may be related to their diet. In this experiment, the fish were fed with alfalfa during experimental periods. Alfalfa is a source of proteins, natural antioxidants (xanthophyll and its derivatives), flavonoids, isoflavonoids, coumestans and lignans, vitamins (C, D, E, K, and P) and minerals (especially iron and copper) [16, 17, 18, 19, 20]. Xie *et al.* [21] showed that the amino acid profile of alfalfa has high antioxidant properties. Pandey and Rizivi [22] suggested that flavonoids could boost cellular antioxidant systems. Their results showed that flavonoids increase the rate of glutathione synthesis and conversion rate of GSSG to GSH in cells. Increased GSH levels and total antioxidant capacity were observed in liver tissue of fish exposed to tetrachloride carbon after treatment with *Astragalus* polysaccharides [23] and silymarin [24, 25].

Prevention of lipid peroxidation in liver and kidney tissues confirmed that the ability of cellular antioxidant defense system has increased. However, MDA levels, as lipid peroxidation biomarker, were significantly increased in gills tissue of fish after exposure to 15 mg/L atrazine at the end of experimental periods. Treatment of carp exposed to carbon tetrachloride with licorice, *Glycyrrhiza glabra*, [26] and milk thistle, *Silybum marianum*, [24] returned the levels of MDA to normal levels. In contrast, Xing *et al.* [27] found that MDA levels in kidney increased after carp exposure to atrazine.

Significant increase in CAT activity levels in kidney and liver tissue of fish exposed to 15 mg/L atrazine on day 10 and 20 of the trial might be biochemical responses to over production of H_2O_2 in liver and kidney's cells. Increase in CAT activity was reported in liver tissue of rainbow trout exposed to atrazine [10]. Our results are similar to results from other researchers [28, 29]. Regulation and return of catalase activity to normal levels may reflect effects of alfalfa's flavonoids on reducing level of ROS produced in the atrazine metabolism process. The protective effect of the flavonoids derived from various plant sources have been reported on tissue injury induced by a diverse group of xenobiotics in experimental animals [30, 31, 32, 33].

5 Conclusion

Since grass carp are fed with plant sources, their antioxidant defense system may be more effective in

reducing the effects of oxidative stress compared with other species. Our results show that feeding the fish with fresh diets of natural origin (alfalfa) which is full of flavonoids, natural antioxidants and vitamins, may have a considerable influence on the physiological condition and thus the homeostasis of the fish body. Our study suggests that diet may impact fish physiological response to oxidative stress caused by exposure to Atrazine.

Acknowledgements

Special thanks are extended to research assistant of Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz for their financial support.

Reference

1- M. Afkhami, M. Shariat, N. Jaafarzadeh, H. Ghadiri and R. Nabizadeh, Developing a water quality management model for Karun and Dez Rivers. Iran. J. Environ. Health. Sci. Eng., 4 (2007) 99-106.

2- M. Banaee, Adverse effect of insecticides on various aspects of fish's biology and physiology, In: S. Soloneski and M. Larramendy, Insecticides – Basic and Other Applications Book, Published by InTech, 2012, pp. 101-126.

3- R. Dahmardeh Behrooz, A. Esmaili-Sari, S.M. Ghasempouri, N. Bahramifar and A. Covaci, Organochlorine pesticide and polychlorinated biphenyl residues in feathers of birds from different trophic levels of South-West Iran. Environment International 35 (2009), 285–290.

4- A. Behfar, Z. Nazari, M.H. Rabiee, G.H. Raeesi, M.R. Oveisi, N. Sadeghi and B. Jannat, The organochlorine pesticides residue levels in Karun River Water. Jundishapur Journal of Natural Pharmaceutical Products, 8 B (2013), 41-46.

5- R. Jin and J. Ke, Impact of Atrazine Disposal on the Water Resources of the Yang River in Zhangjiakou Area in China. Bulletin of Environmental Contamination and Toxicology, 68 (2002), 893-900.

6- L. Spanó, C.R. Tyler, R. van Aerle, P. Devos, S.N.M. Mandiki, F. Silvestre, J.P. Thomé, and P. Kestemont, Effects of atrazine on sex steroid dynamics; plasma vitellogenin concentration and gonad development in adult goldfish (*Carassius auratus*). Aquatic Toxicology, 66 (2004), 369–379.

7- S.Y. Hussein, M.A. El-Nasser and S.M. Ahmed, Comparative Studies on the Effects of Herbicide Atrazine on Freshwater Fish *Oreochromis niloticus* and *Chrysichthyes auratus* at Assiut; Egypt. Bulletin of Environmental Contamination and Toxicology, 57 (1996), 503-510.

8- A.C. Elia, W.T. Waller and S.J. Norton, Biochemical Responses of Bluegill Sunfish (*Lepomis macrochirus*; Rafinesque) to Atrazine Induced Oxidative Stress. Bulletin of Environmental Contamination and Toxicology, 68 (2002), 809-816.

9- M.T. Wan, C. Buday, G, Schroeder, J. Kuo and J. Pasternak, Toxicity to *Daphnia magna*, *Hyalella azteca*, *Oncorhynchus kisutch*, *Oncorhynchus mykiss*, *Oncorhynchus tshawytscha*; and *Rana catesbeiana* of Atrazine; Metolachlor; Simazine; and Their Formulated Products. Bulletin of Environmental Contamination and Toxicology, 76 (2006), 52-58.

10- I. Salaberria, B.H. Hansen, V. Asensio, P.A. Olsvik, R.A. Andersen and B.M. Jenssen, Effects of atrazine on hepatic metabolism and endocrine homeostasis in rainbow trout (*Oncorhynchus mykiss*). Toxicology and Applied Pharmacology, 234 (2009), 98–106.

11- E.D. Hahn and R. Soyer, "Probit and Logit Models: Differences in a Multivariate Realm." Retrieved May 28; 2008; from http://home.gwu.edu/~soyer/mv1h.pdf.

12- F. Iris, F. Benzie and J.J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of ''antioxidant power'': the FRAP assay. Analytical Biochemistry, 239 (1996), 70–76.

13- E. Beutler, O. Duron and B.M. Kelly, Improved method for the determination of blood glutathione. J. Lab. Clin. Med. 61 (1963), 882-888.

14- Z.A. Placer, L. Cushman and B.C. Johnson, Estimation of products of lipid peroxidation (malonyl dialdehyde) in biological fluids. Analytical Biochemistry, 16 (1966), 359-364.

15- L. Góth, A simple method for determination of serum catalase and revision of reference range. In: Clinica Chimica Acta, 196 (1991), 143-152.

16- A.E. Khaleel, M.Z. Gad, S.A. El-Maraghy, M.S. Hifnawy and E. Abdel-Sattar, Study of hypocholesterolemic and antiatherosclerotic properties of *Medicago sativa* L. cultivated in Egypt. Journal of Food and Drug Analysis, 13 (2005), 212−218.

17- P. Avato, R. Bucci, A. Tava, C. Vitali, A. Rosato and Z. Bialy, Antimicrobial activity of saponins from *Medicago* sp.: Structure–activity relationship. Phytotherapy Research; 20 (2006); 454−457.

18- B.P. Lamsal, R.G. Koegel and S. Gunasekaran, Some physicochemical and functional properties of alfalfa soluble leaf proteins. LWT Food Science and Technology; 40 (2007), 1520−1526.

19- E.R. Grela, V. Semeniuk and M. Florek Effects of protein-xanthophyll (PX) concentrate of alfalfa additive to crude protein-reduced diets on nitrogen excretion; growth performance and meat quality of pigs. Journal of Central European Agriculture, 9 (2008), 669−676.

20- M. Karwowska, J. Stadnik, Z.J. Dolatowski and E.R. Grela, Effect of protein-xanthophylls (PX) concentrate of alfalfa supplementation on physico-chemical properties of turkey breast and thigh muscles during ageing. Meat Science, 86 (2010), 486–490.

21- Z. Xie, J. Huang, X. Xu and Z. Jin, Antioxidant activity of peptides isolated from alfalfa leaf protein hydrolysate. Food Chemistry, 111 (2008), 370–376.

22- K.B. Pandey and S.I. Rizvi, Plant polyphenols as dietary antioxidants in human health and disease. Oxid. Med. Cell. Longev., 2 (2009): 270–278.

23- R. Jia, L. Cao, P. Xu, G. Jeney and G. Yin, In vitro and in vivo hepatoprotective and antioxidant effects of *Astragalus* polysaccharides against carbon tetrachloride-induced hepatocyte damage in common carp (*Cyprinus carpio*). Fish Physiology and Biochemistry; 38 (2012): 871–881.

24- R. Jia, L. Cao, J. Du, P. Xu, G. Jeney and G. Yin, The protective effect of silymarin on the carbon tetrachloride (CCl4)-induced liver injury in common carp (*Cyprinus carpio*). In Vitro Cell. Dev. Biol.-Animal, 49 (2013), 155–161.

25- M. Banaee, A. Sureda, S. Shahaf and N. Fazilat, Protective Effects of Silymarin Extract on Malthion-Induced Zebra Cichlid (*Cichlasoma nigrofasciatum*)

Hepatotoxicity. Iranian Journal of Toxicology, (2015), (*Article in Press*).

26- G. Yin, L. Cao, L. Cao, P. Xu, G. Jeney, M. Nakao and C. Lu, Hepatoprotective and antioxidant effects of *Glycyrrhiza glabra* extract against carbon tetrachloride (CCl4)-induced hepatocyte damage in common carp (*Cyprinus carpio*). Fish Physiology and Biochemistry, 37 (2011), 209–216.

27- H. Xing, S. Li, Z. Wang, X. Gao, S. Xu and S. Wang, Histopathological changes and antioxidant response in brain and kidney of common carp exposed to atrazine and chlorpyrifos. Chemosphere, 88 (2012), 377–383.

28- D.A. Monterio, J.A. Almeida, F.T. Rantin and A.L. Kalinin, Oxidative stress biomarkers in the freshwater characid fish; *Brycon cephalus*; exposed to organophosphorus insecticide Folisuper 600 (methyl parathion). Comp. Biochem. Physiol. C 143 (2006), 141–149.

29- M. Banaee, A. Sureda, A.R. Mirvaghefi and K. Ahmadi, Biochemical and histological changes in the liver tissue of Rainbow trout (*Oncorhynchus mykiss*) exposed to sub-lethal concentrations of diazinon. Fish Physiology and Biochemistry, 39 (2013), 489-501.

30- S. Mahaboob Khan, R.C. Sobti and L. Kataria, Pesticide-induced alteration in mice hepato-oxidative status and protective effects of black tea extract. Clinica Chimica Acta, 358 (2005), 131–138.

31- C. Nencini, G. Giorgi and L. Micheli, Protective effect of silymarin on oxidative stress in rat brain. Phytomedicine, 14 (2007), 129–135.

32- L.P. Yuan, F.H. Chen, L. Ling, P.F. Dou, M.M. Zhong and L.J. Xia, Protective effects of total flavonoids of *Bidens pilosa* L. (TFB) on animal liver injury and liver fibrosis. Journal of Ethnopharmacology, 116 (2008), 539–546.

33- M. Banaee, Influence of silymarin in decline of sublethal diazinon-induced oxidative stress in rainbow trout (*Oncorhynchus mykiss*). Ph.D. Thesis; Aquaculture and Environmental Department; Natural Resource Faculty; Natural Resource and Agriculture Collage; Tehran University, Iran, (2010), 149 pages.