APRIL 2017 M.Sc. in Biochemistry Science and Technology AKRAM MUDHER KAREEM ALJBORI

UNIVERSITY OF GAZİANTEP

GRADUATE SCHOOL OF

NATURAL & APPLIED SCIENCES

EFFECT ON LEVELS OF PLASMA PARAOXONASE ACTIVITY, HIGH DENSITY LIPOPROTEIN AND MALONDIALDEHIDE OF BELLIS IN *CYPRINUS CARPIO*

M.Sc. THESIS

IN

BIOCHEMISTRY SCIENCE AND TECHNLOGY

BY

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Supervisor

Assist. Prof. Dr. H. Ahmet DEVECİ

Co-Supervisor

Assist. Prof. Dr. Gökhan NUR

by

Akram Mudher Kareem ALJBORI

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Name of the student: Akram Mudher Kareem ALJBORI

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Approval of the Graduate School of Natural and Applied Sciences

Prof. Dr. Ahmet Necmeddin YAZICI

Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

Prof. Dr. Canan CAN

Head of Department

This is to certify that we have read this thesis and that in our majority opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Assist. Prof. Dr. Gökhan NUR
Co-Supervisor

Assist. Prof. Dr. H. Ahmet DEVECİ
Supervisor

Examining Committee Members	Signature
Prof. Dr. Canan CAN	.....
Assist. Prof. Dr. H. Ahmet DEVECİ	.....
Assist. Prof. Dr. Zuhal UÇKUN	.....

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Akrem Mudher Kareem ALJBORI

ABSTRACT

EFFECT ON LEVELS OF PLASMA PARAOXONASE ACTIVITY, HIGH DENSITY LIPOPROTEIN AND MALONDIALDEHIDE OF BELLIS IN *CYPRINUS CARPIO*

ALJBORI, AKRAM MUDHER KAREEM

M.Sc. in Biochemistry Science and Technology

Supervisor: Assist. Prof. Dr. H. Ahmet DEVECİ

Co-Supervisor: Assist. Prof. Dr. Gökhan NUR

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One of the most important causes of environmental pollution is pesticides widely used in agriculture . In this study, 24 fish (200-300 g) *Cyprinus carpio* from Islahiye Tahtakopru Dam were used. The fish were transported to the laboratory in polyethylene tanks for a period of 5 days (22–25 °C and normal photoperiod). Then they were divided into 3 groups of 24 fishes: A control group, 0.025 mg/L Bellis I and 0.050 mg/L Bellis II. After the end of 14 days of application, blood samples were taken from the fishes and the plasma were separated . Paraoxonase (PON1) activity, high density lipoprotein (HDL) and Malondialdehide (MDA) were analyzed in the obtained plasma samples. PON1 and HDL levels in control group was found statistically significantly higher than Bellis I and Bellis II group ($p<0.001$) While MDA level in control group was found Statistically significant lower than Bellis I and Bellis II group ($p<0.05$).

In conclusion, it is thought that Bellis may causes changes in PON1 enzyme activity, HDL and malondialdehyde levels which are involved in the detoxification of pesticides in fish.

Keywords: Paraoxonase, Malondialdehide, High density lipoprotein, Bellis, *Cyprinus carpio.*

ÖZET

SAZAN BALIĞINDA BELLIS'IN PLAZMA PARAOKSONAZ AKTİVİTESİ, YÜKSEK DANSİTELİ LİPOPROTEİN VE MALONDİALDEHİT SEVİYELERİ ÜZERİNE ETKİSİ

ALJBORI, AKRAM MUDHER KAREEM

Yüksek Lisans Tezi, Biyokimya Bilimi ve Teknolojisi

Danışman: Yrd. Doç. Dr. H. Ahmet DEVECİ

İkinci Danışman: Yrd. Doç. Dr. Gökhan NUR

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Çevre kirliliğinin en önemli nedenlerinden biri tarım alanında yaygın bir şekilde kullanılan pestisitlerdir. Bu çalışmada, İslahiye Tahtaköprü Barajı'ndan tutulan 24 adet (200-300g) *Cyprinus carpio* kullanılmıştır. Balıklar polietilen tanklar içerisinde 5 günlüğüne laboratuvara getirilip muhafaza altına alındı (22-25°, normal fotoperiyod). Daha sonra balıklar; kontrol grubu, 0.025 mg/L Bellis I ve 0.050 mgl/L Bellis II olmak üzere 3 gruba ayrıldı. 14 günlük fungusit uygulamasının ardından balıklardan alındı ve plazmaları ayrıldı. Paraoksonaz (PON1), yüksek dansiteli lipoprotein (HDL) ve Malondialdehit elde edilen plazma numunelerinde çalışıldı. Yapılan çalışma neticesinde kontrol grubunda PON1 ve HDL düzeyleri Bellis I ve Bellis II grubundan anlamlı şekilde daha yüksek bulunmuştur ($p<0.001$). Buna karşın kontrol grubunda MDA düzeyi ise Bellis I ve Bellis II grubundan anlamlı şekilde daha düşük bulunmuştur ($p<0.05$). Sonuç olarak yapılan çalışma sonucunda son zamanlarda kullanılmaya başlanan etkili bir fungusit olan Bellis'in sazan balıklarında pestisitlerin detoksifikasyonunda görev alan PON1 enzim aktivitesi, HDL ve Malondialdehit düzeylerinde değişikliğe neden olabileceği düşünülmektedir.

Anahtar Kelimeler: Paraoksonaz, Malondialdehit, Yüksek dansiteli lipoprotein, Bellis, *Cyprinus carpio.*

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LIST OF ABBREVIATIONS

AC : After Christ

BC : Before Christ

DDT : Dichlorodiphenyltrichloroethane

HCS : Hexachlorocyclohexane

MDA : Malondialdehide

HDL : High Density Lipoprotein

IDL : Intermediate Density Lipoprotein

LDL : Low Density Lipoprotein

OCP : Organochlorine Pesticides

PBDE : Polybrominated Diphenyl Ethers

PCDD : Polychlorodibenzodioxins

PCDF : Polychlorodibenzofuran

PON1 : Paraoxonase

BHC : Beta-hexachlorocyclohexane

BHT : Butulated Hidroxy Toluen

TBA : Thiobarbituric Acid

CHAPTER I

INTRODUCTION

1.1. Pesticides

1.1.1. Definition and Classification

Pesticides, which are chemical and biological substances used in the struggle with the so-called 'pests', are widely used in veterinary medicine and agricultural struggle for protective purposes against internal and external parasites (Ekebaş et al., 2000). Pesticides are often used to control crops and insects that damage crops and disease-affecting vectors in order to obtain more crops (Abdollahi et al., 2004).

Pesticides applied in very low amounts from air can reach the food chain by going to waters and soils and then to plants and animals. The accumulation of the residue occurred by feeding bigger organisms to smaller ones that live in the water seen in a much higher and concentrated state in the human that found in the last step of the food chain. Gradually increment of that residue in the food chaing and in the living things called as bioconcentration (Ceron et al., 1995; Kaya, 2005). The harmful effects of pesticides on humans and animals are twofold. First; acute poisonings caused by the direct ingestion of pesticides into the body, second one; chronic poisonings that occurs depending on pesticide production and the studies in application areas, or repeatedly in take of residues in foods that contains small quantities. When pesticides are taken in the body, they are metabolized by enzymes and thrown out. However, certain parts cause chronic poisonings by accumulation (Blanco-Coronado et al., 1992; Kaya, 2005).

There are traces about the pesticides being used in ancient times. Records had been found in a papyrus belongs to 1500 B.C showing preparation of pesticides against wasps, fleas and lice (Miller, 2002; Altıkat et al., 2009).

Pesticide residues became important for the first time in 1948 with the encountering organochlorine pesticide residues in human body. While some of the pesticides have toxic effects, it has also been found that some of them carcinogenic and have

negative effects on the nervous system (Akman et al., 2004; Altıkat et al., 2009). Most of the settlements in Turkey provide drinking water from wells and soundings. The pollution of underground water is therefore important for people. The pesticide residues in the water generally remain insoluble; they remain on organic matter, clay, sediments, decaying debris and planktons as suspension. In this way they enter the food chain and accumulate easily in fishes, invertebrates living in aquatic environment. Insecticide residues adhering to planktons and bacteria in the aquatic environment enter the food chain up to fish; reach the highest density in fishes. In fish-fed organisms, this density reaches higher levels. Young fish are much more sensitive and delicate to some pesticides. Since many fish species have a very low viability at this point in their life cycle, the harmful effects of agricultural chemicals further reduce the numbers of these organisms (Altıkat et al., 2009; Oden, 2009).

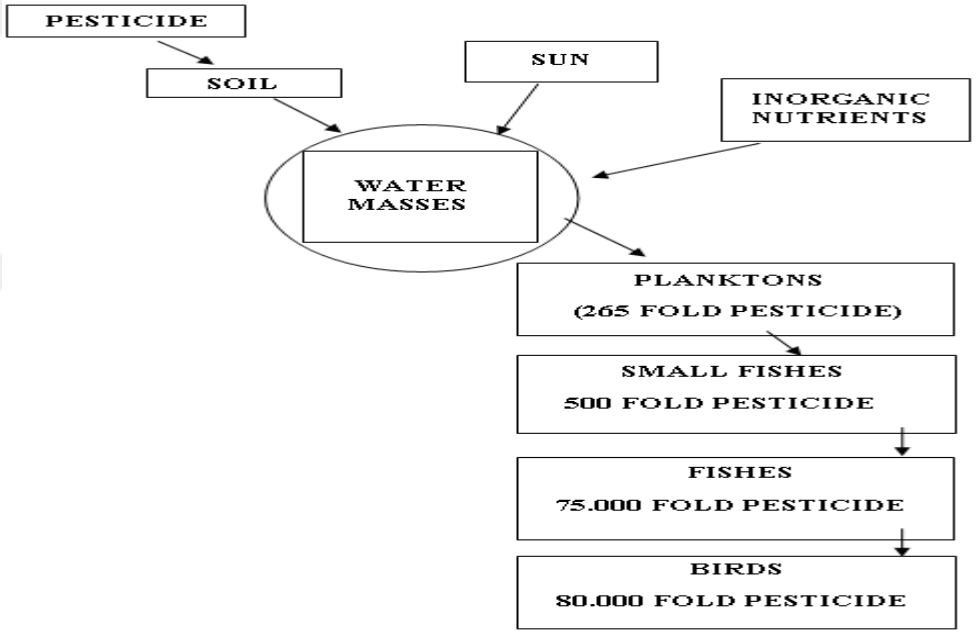

Figure 1.1. Biological accumulation of pesticides (Güler and Cobanoğlu, 1997).

Especially in recent years agricultural and industrial chemicals have been mixed to aquatic ecosystems increasingly. Taking these chemicals by aquatic organisms occur by water, sediment, suspended particles and nutrients. Environmental pollutant chemicals taking into metabolism by the organisms can disrupt metabolic processes that play a role in fighting with free radicals (Halliwell and Gutteridge., 1989; Akbulut et al., 2014). The widespread use of pesticides used for 3000 years has been

mainly occurred since the 1940s. After that date the activities have increased more. However, the fact that the pesticides used against harmful organisms also have a negative impact on useful arthropods, which in turn hinders the biological warfare with harmful insects, has led people to turn to environmentally friendly methods of struggle (Romoser and Stoffolano, 1994; Akkuzu et al., 2001).

Pesticides are classified according to their target species as follows (Kurutaş and Kılınç, 2003):

a) Fungicide: Fungi killer

b) Insecticides: Insect killer

c) Acaricides: Mites killer

d) Nemacides: Nematode killer

e) Mollucides: Clams killer

f) Rodenticides: rodent killer

g) Avicides: Bird killer

h) Aphicides: Aphids killer

i) Bactericidal: bacterial killer

j) Herbicides: Weed killer

k) Algaecides: Algae killer

1.1.2. The Effects of Pesticides on Environment

Pesticides and other chemicals are not only a potential risk for humans, but also for the environmental population in which the general environment and chemicals are used (www.ilo.org/global, 2016).

The ecological effects of pesticides can be extended to the ecosystem beyond individual organisms (www.fao.org, 2016). The effects of pesticides on the ecosystem can be considered as side effects on non-target organisms and the

movement of pesticides in the environment (Chakravarty, 2014). The behavior of pesticides in the ecosystem (soil-plant-environment-atmospheric system) is shown in Figure 1.2.

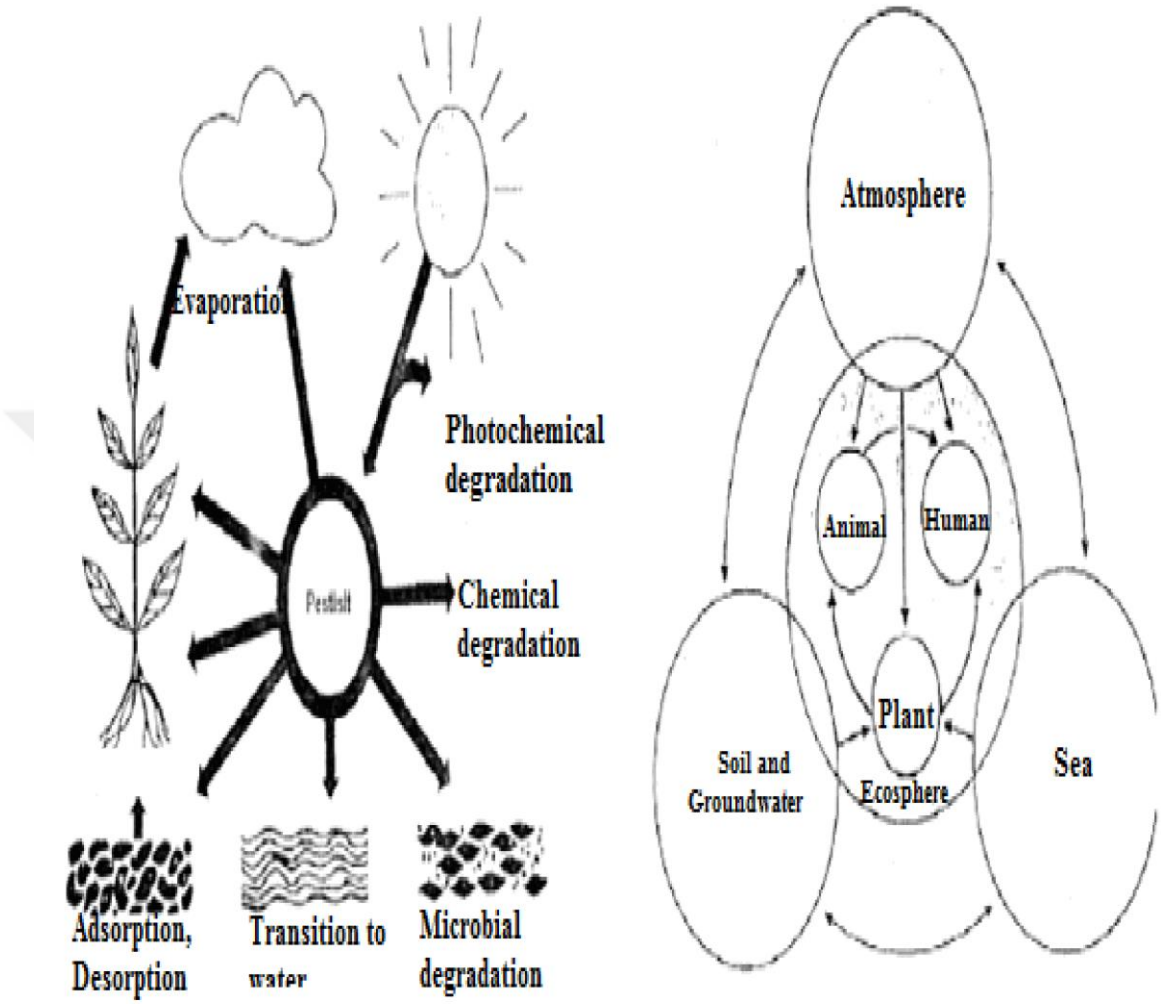

Figure 1.2. Behavior of pesticides in ecosystem (Tiryaki et al., 2010).

In places where pesticides are used, soils are transported to areas far away from the places they are, such as polluted water, by biological and physical means (Vural, 2005). The movement of a pesticide is effected from its chemical, physical properties, formulation type, application type, climate and agricultural conditions (megep.meb.gov.tr, 2016). Especially those that are resistant in the environment (slower biological degradation rates) and soluble in lipids are detrimental to all living things (bio-cumulation and bioconcentration) by accumulation in the bio-ecosystem (Vural, 2005).

Fisheries are essential part of the diet in most countries and constitute the main source of functional compounds and proteins that are very important for human health. On the other hand, aquatic products generally have an active role in the transmission of contaminants such as organochlorine compounds (OCPs), polychlorodibenzodioxins (PCDDs), polychlorodibenzofuran (PCDFs), dioxins, polybrominated diphenyl ethers (PBDEs) and heavy metals (mercury, arsenic, cadmium, lead) via food to the human. Especially species with high fat concentration are the most risky food groups. These lipophilic contaminants accumulate in the fatty tissues of the aquatic products and shift to humans through the food chain and have adverse effects on health. In recent years, there has been an increase in attention to this issue from official and non-formal institutional establishments due to the widespread consumption of aquatic products all over the world, and in the studies, the remnants of these substances have been encountered in water products (Ozçelik et al., 2011). Environmental contamination of natural waters around the world with pesticide residues causes great concern. Pesticides mix with rain water and erosion into the water ecosystem. Some soluble pesticides more easily reach these sites and accelerate contamination (Pimentel et al., 1992; Bansal, 2012).

Pesticide residues entering by the ways mentioned above are also entering to an organism living in the sea or water (Kaya, 2007). Pesticide residues entered an organism in this way, accumulate in the animal tissue and enter from one organism to another via the food chain (Barlas et al., 2000; Harvey et al., 2008). The biggest depositors of these organic pollutants are the oceans and seas. Persistent organic pollutants (POPs) are stored in sediments in the sea bottom and are transferred through water and the food chain in the atmosphere. A number of studies show that all underground and surface waters in the world are contaminating with pesticides and their derivatives. Although the use of DDT and Beta-hexachlorocyclohexane (BHC) has been terminated in the last decade in the world, these pesticides are found above the permissible values, especially in developing countries. Pesticide and its derivatives transmitted from household, industrial and agricultural wastes are also transferred to food chain through surface and underground waters (Bansal, 2012; Hongsheng, 2011).

Qualitative and quantitative changes happen within bioavailability and bioaccumulation depend on various biotic (habitat, food chain status, detoxification

mechanisms, sexual maturity/physical factors, gender, fat content, nutritional pattern, tissue composition and metabolic capacity) and abiotic (pysical and chemical properties of chemicals, environmental factors such as light, dissolved oxygen concentration, salinity, pH, and temperature). While these factors alone are not enough, there may be differences in residual levels depending on what species and contaminants are (Ozçelik et al., 2011).

The distribution and transportation of the pesticides in water depends on both their chemical structure and formulations in addition to environmental conditions. Some pesticides are soluble in water and homogeneously dispersible, while some inorganic salts are precipitated in water. The absorption of the pesticides by organisms living in the water depends on pesticide level in the water, the physiology of the organism, the temperature and pesticide residues existing previously in the structure of the organism (Atamanalp, 2004; Mahmoud and Loutfy, 2012).

Pesticide residue concentrations are 1000-10000 times more in fish than in water. Although consumption of fish and aquatic products is very beneficial for human health, these products are an important source of human intake of these chemicals. In this context, fish is considered as an indicator because it can contain more than these substances. The amount of pesticide residues varies according to the species and size of the fish (Barlas et al., 2000; Aktümsek et al., 2002; Storelli et al., 2004). Consuming foods contaminated with pesticides is a very dominant way to get these things into the body. In some studies it has been reported that there is a significant correlation between organochlorine concentrations in humans and fish consumption (Raeside, 2007).

The risks related to pesticide usage can be divided into two groups (Tijani, 2006):

1. Risks against humans include risks such as toxic effects, carcinogenic effects and biological concentrations that can be categorized acutely and chronically. The exposure of people to pesticide is a major health and social issue. Because it can often result in serious health problems such as epilepsy, stroke, respiratory diseases, cancer, leukaemia, brain and liver tumours, convulsions. It is known that in some places death occur due to pesticide exposure.

2. Environment-related risks occur as a result of abnormalities and contamination in the ecosystem. Surface waters, groundwater, soil and air can be contaminated by pesticides.

1.2. Bellis

It is a fungicide transported by translaminar and locally-systemic with protective and preventive effect. It prevents spore germination, germination tube and micelle development and spore formation. It contains 12.8% pyraclostrobin and 25.2% boscalid. (http://www.agro.basf.co.za, 2016).

1.2.1. Pyraclostrobin

Pyraclostrabin is a fungicide belonging to the group known as strobilurins that inhibit mitochondrial respiration. In this context it blocks the energy supply of the cell.

ISO common name: Pyraclostrobin

Chemical name: Methyl *N*-{2-[1-(4-chlorophenyl)-1*H*-pyrazol-3-yl] oxymethyl] phenyl} (*N*methoxy) carbamate.

Formula: The chemical formula of pyraclostrobin is seen in Figure 1.3.

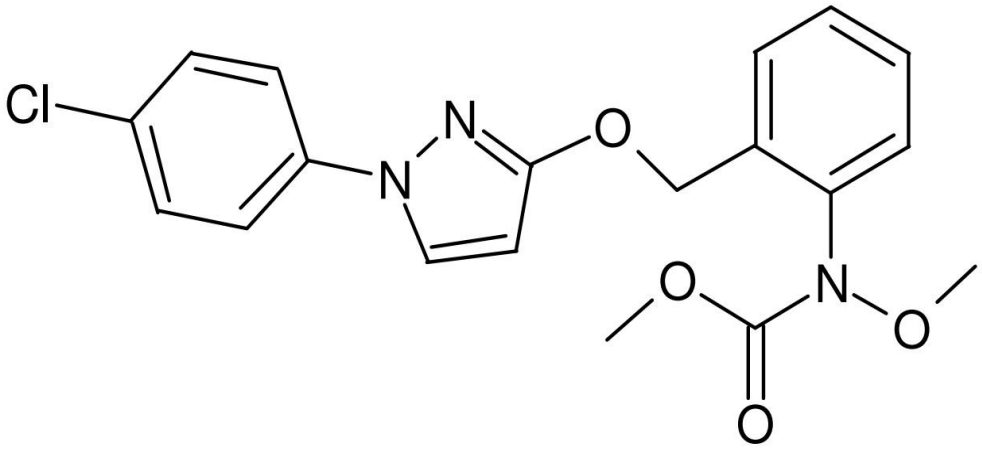

Figure 1. 3. The chemical structure of pyraclostrobin (www.fao.org, 2016).

1.2.2. Boscalid

It is fungicide belonging to the carboxamides. It is fat soluble. Boscalid controls several fungal pathogens belonging to 4 main classes of plant pathogenic fungi effectively. It is envisaged to be used as fungicide on several agricultural and horticultural, ornamentals and viticulture. It has preventative and curative properties.

ISO Common Name: Boscalid

Chemical Name: Chemical name IUPAC name: 2-Chloro-N-(4'-chlorobiphenyl-2-yl) nicotinamid

Formula: The chemical formula of boscalid is seen in Figure 1.4.

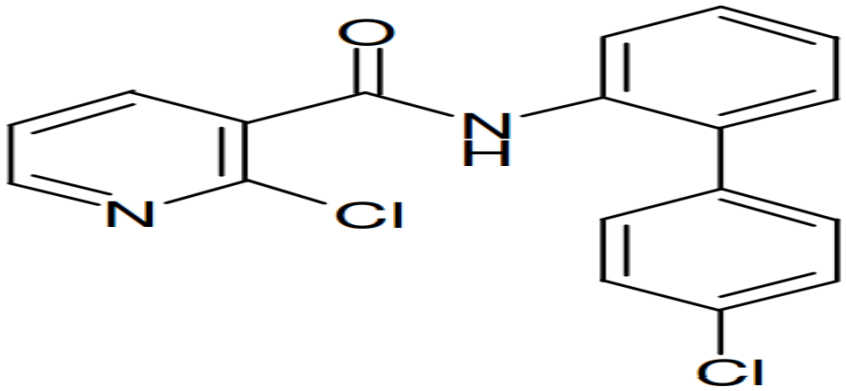

Figure 1.4. The chemical structure of boscalid (pubchem.ncbi.nlm.nih.gov, 2016) .

1.3. Paraoxonase Enzyme (PON, EC 3.1.8.1)

Paraoxonase enzyme (PON) was first discovered in 1953 by Aldridge W.N. (1983) as A-esterases which hydrolyze pnitrophenyl acetate, propionic acid and butyric acid. In 1965 Oooms and Booter (1965), parathion and paraoxon-specific hydrolysis properties were determined. In 1973, a group of German researchers first genetically detected human serum PON (Geldmacher et al., 1973). Until 1985, PON was prufied and the effect on neurotoxic organophosphates and its role in detoxification were investigated (Brophy et al., 2001). Since it has the property of hydrolyzing the metabolite “paraoxon” which is the metabolite of parathion (it is an insecticide) the name “paraoxonase” was given in 1985 (Noelker et al., 2005). In 1985, Mackness et al. (1985) first discovered enzyme-arylase activity of the enzyme in the lipoprotein

fraction during HDL cholesterol differentiation. In 1988, Mackness et al. (1988) found that PON activity was dependent on apoA1 on HDL and decreases lipoperoxide accumulation on LDL. By immunoaffinity chromatography in 1993-1994, PON was found to be associated with apo A1 and clusterin (apo J) components of HDL (Nug et al., 2005). In 1996, it was found that the gene responsible for paraoxonase activity is a member of a multigen family and named respectively as PON1, PON2 and PON3 (Primo-Parmo et al., 1996).

Although paraoxonase and arylesterase are considered as two separate enzymes, it is reported that PON1, the only gene product in human serum, has both arylesterase and paraoxonase activities (Başkol and Köse, 2004) .When paraoxon is used as the substrate, the paraoxonase activity of the enzyme; when phenylacetate is used as the substrate, the arylesterase activity of the enzyme is measured. It is even present in studies showing that this enzyme also has lactonease activity (Başkol and Köse, 2004; Eckerson et al., 1983).

1.3.1. Structural Properties of Paraoxonase

Paraoxonase (PON1) is a glycoprotein structural protein with 43-45 kDa molecular weight, includes 354 amino acids and its activity and stability depends on Ca^{+2} ion. Each molecule contains four carbohydrate chains that constitute 15.8% of the total weight. Isoelectric point pH is 5.1. This enzyme, which catalyzes the hydrolysis of organophosphates, is common in the liver, kidney, intestine and serum attached to HDL (Watson et al., 1995).

PON1 is easily able to bind to HDL lipids via the hydrophobic N-terminal region. Since HDL subunits contain also Apolipoprotein A1 (ApoA1) and Apo J (Clusterin) proteins, it is thought that Apo A1 and Apo J may play a role in binding (Deakin and James, 2004).

As seen in Figure 1.5, two polymorphic areas were indicated in 55. and 192. positions. The present carbohydrate chains and the hydrophobic head are shown at the amino terminal end of the chain (Aviram, 1999).

PON1 has been placed in a helix form and consists of 6 β layers, each consisting of 4 rows. The structure of PON1 has three hydrophobic helix structures (H1, H2 and

H3). The H2 and H3 hydrophobic helixes are present in the active site and have critical roles such as the determination of the structure of the active site, binding of the enzyme to HDL (Eckerson et al., 1983).

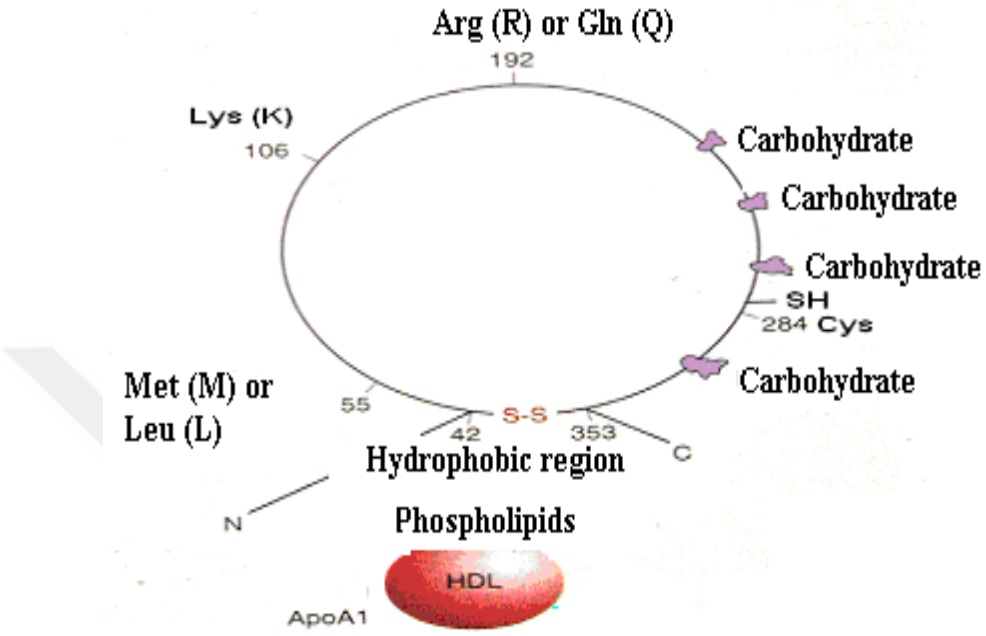

Figure 1.5. The structure of paraoxonase enzyme (Aviram, 1999).

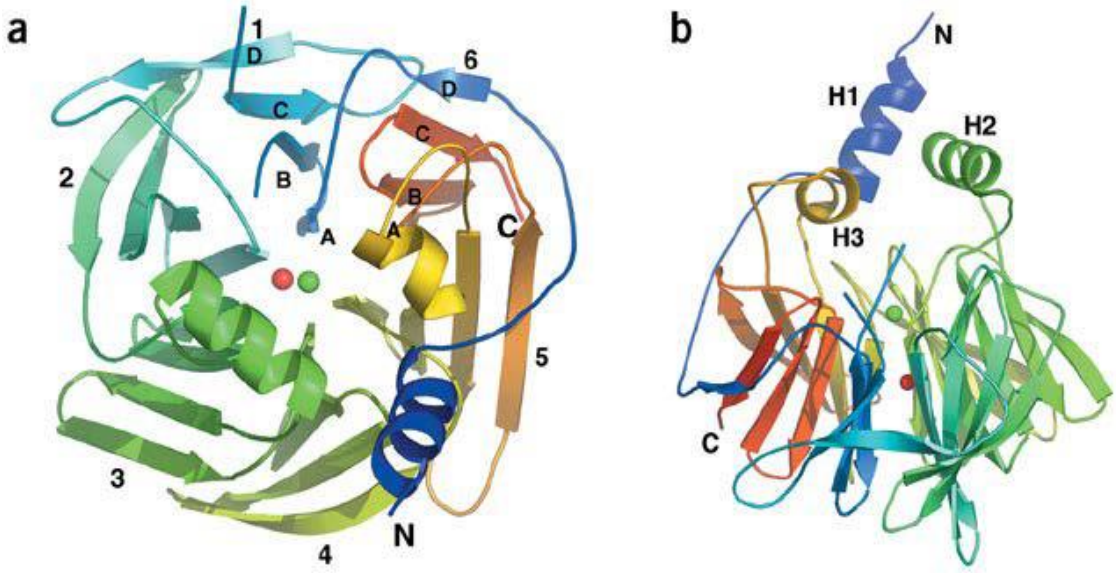

Figure 1. 6. Three dimensional structure of PON1 (Josse et al., 1999)

a- Top view of 6-ß helix structure

b- Side view of 6-ß helix structure

1.3.2. Biological Functions of Paraoxonase

The best-known biological function of PON1; to prevent neurotoxicity of organophosphates entered into circulation by binding oppositely to organophospahates such as nerve gases (soman, sarin), aromatic carboxylic acid esters and insecticides. Paraoxon (O, O-diethyl-O-p-nitrophenyl phosphate) which is the active catabolic metabolite of parathion is a strong inhibitor of cholinesterase that degrades acetylcholines. The hydrolytic products comprised by the effect of PON1 to paraoxon are less harmful than paraoxonase itself. It has seen that compared to the mammals, inclination to organophosphate poisoning due to the absence of PON1 is higher in invertebrates, birds and fishes (Costa et al., 2000; Costa et al., 2003).

The calcium ion involved in the catalytic activity interacts with the oxygen of the phosphate ion in the presence of a water molecule and facilitate the separation of diethyl phosphate from active site by polarizing the paraoxone P = O bond. While the hydrolytic activity of PON1 against organophosphate substrates is dependent on two calciums, it is reported that only structural calcium is sufficient to prevent the accumulation of lipid peroxides (Lourdes et al., 2001).

The second biological function of PON1 is antiatherogenic activity. Serum PON1 enzyme is associated with HDL in the plasma and is thought to play a role in the prevention of the oxidation of plasma lipoproteins. Since peroxidased lipids are metabolized by this enzyme, accumulation of lipid peroxides in both HDL and LDL is prevented. The protective effect of HDL on LDL against oxidation is more effective than A and E vitamins (Rousselot et al., 1999).

Because of being antioxidant enzyme, PON1 is thought to play a protective role against many diseases such as cardiovascular diseases, diabetes, sepsis, Alzheimer and Parkinson (Draganov and LaDu, 2004).

The natural activity of PON1 has been reported to be lactonose and in recent publications this enzyme is defined as calcium dependent lipophilic lactonose. PON1 shows its protectivity against atherosclerosis with its lactonos activity by especially preventing the oxidation of LDL and HDL lipids, metabolizing fatty acid oxidation in HDL and LDL (Khersonsky and Tawfik, 2006).

1.4. Plasma Lipids

The essential lipids in the plasma are; cholesterol, triglyceride, phospholipids and free lipid acids. One of the most characteristic features of lipids is their low solubility in the water. The special structures called as lipoproteins increase the solubility of lipids in the water and provide the transportation in the plasma. The molecules with protein structure and associated with lipoproteins located on their surface are called as "*apolipoprotein*". Their task is to bind lipoproteins to the receptors on the cell surface, to stabilize apolar lipids and to metabolize lipoproteins. A lipoprotein consists of a outer layer composed of protein and polar lipids (phospholipid and free cholesterol) and nucleus composed of hydrophobic and neutral lipids (triglyceride and cholesterol ester) (Rifai et al., 1999).

Cholesterol, the main sterol in organism, is the precursor of all steroids. Phospholipids are composed of as a result of the esterification of two of the hydroxyl groups of a glycerol with fatty acids and the other with phosphoric acid. Triacylglycerols occur when a glycerol molecule and three fatty acid molecules are linked by an ester linkage. Free fatty acids are present in the plasma attached to the albumin. Triglycerides constitute a large part of the storage lipid. Lipoproteins are divided into five groups according to densities; chylomicrons (CM), very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoprotins (LDL) and high-density lipoproteins (HDL) (Mayes, 1993; Rifai et al., 1999; Değer and Orem, 2002).

A. Chylomicrons

These are the largest plasma lipoproteins, containing 98-99% lipid and 1-2% protein. Both phospholipids and cholesterol are used in the formation of large chylomicron particles with triglycerides. VLDLs are formed from chylomicron residues in the liver and these are given circulation.

B. Very Low-Density Lipoproteins

VLDLs containing between 85 and 90 percent lipid and 10-15% protein; in addition to endogenous triglycerides these lipoproteins contain free cholesterol, cholesterol esters, phospholipids and apolipoproteins.

Lipoprotein lipase causes the release of free fatty acids from VLDL triglycerides and the gradually reduction of lipid content in VLDLs transform intermediate-density lipoproteins (IDL) which contain approximately equal amounts of triglyceride and cholesterol, and these transform low-density lipoproteins (LDL).

C. Intermediate-Density Lipoproteins (IDL)

It is normally found in very low concentrations in the plasma. It is between VLDL and LDL in terms of size and content. IDL is the catabolism product of VLDL and precursor of LDL.

D. Low-Density Lipoproteins (LDL)

LDL is the main cholesterol carrier lipoprotein in the plasma, constitutes about 70% of total plasma cholesterol. LDL consisting of approximately 75% lipid and 25% protein, very low triglyceride content and the main apolipoproteins of LDL are apoB-100.

LDLs carry cholesterol to tissues from the liver and transform into cholesterol and its derivatives by breaking down here. Cholesterol and cholesterol derivatives, which occur after the breakdown of LDLs, exhibit various metabolic effects. In the presence of excessive amounts of LDL, LDLs are oxidized by such factors as superoxide and H_2O_2.

E. High-Density Lipoproteins

It is an important class of lipoproteins synthesized in the liver and small intestinal walls. HDLs contain 55% protein, 2% free cholesterol, 15% cholesterol esters, 24% phospholipids and 4% triglycerides. The first mature HDL is known as HDL3.

Later, cholesterol esters increase and HDL2 is formed by addition of apoE and HDL1 is formed in the further stage. HDL, enriched in cholesterol during circulation, releases cholesterol in the liver when it returns to the liver and this function creates an “antiatherogenic effect”.

Lipoproteins, which increase the solubility of lipids in water, also, carry many substances such as fat soluble vitamins (A, D, E, K), drugs (probucol, cyclosporin

etc.) and some antioxidant enzymes (Paraoxonase, platelet activating factor hydrolase-PAFAH) (Değer and Orem, 2002).

Serum PON1 enzyme co-exists with HDL in plasma and is reported to play a role in the prevention of plasma lipoprotein oxidation (Mackness et al., 1985). Experimental studies have shown that the enzyme PON1 is associated with apoA-I and apo J (clustrein) proteins of HDL (Hasselwander et al., 1998). Recently, it has also been found that HDLs contain apoA-II have also been carry PON1 (Deakin and James, 2004). The end region of the hydrophobic structure at the N-terminal end of the PON1 enzyme is required for interaction with HDL. PON1 binds to phospholipids and lipoproteins via this hydrophobic region. ApoA-I is thought to play a role in the binding of the PON1 enzyme to phospholipids. It has also been reported that apoA-I stabilizes PON1 activity (Sorenson et al., 1999).

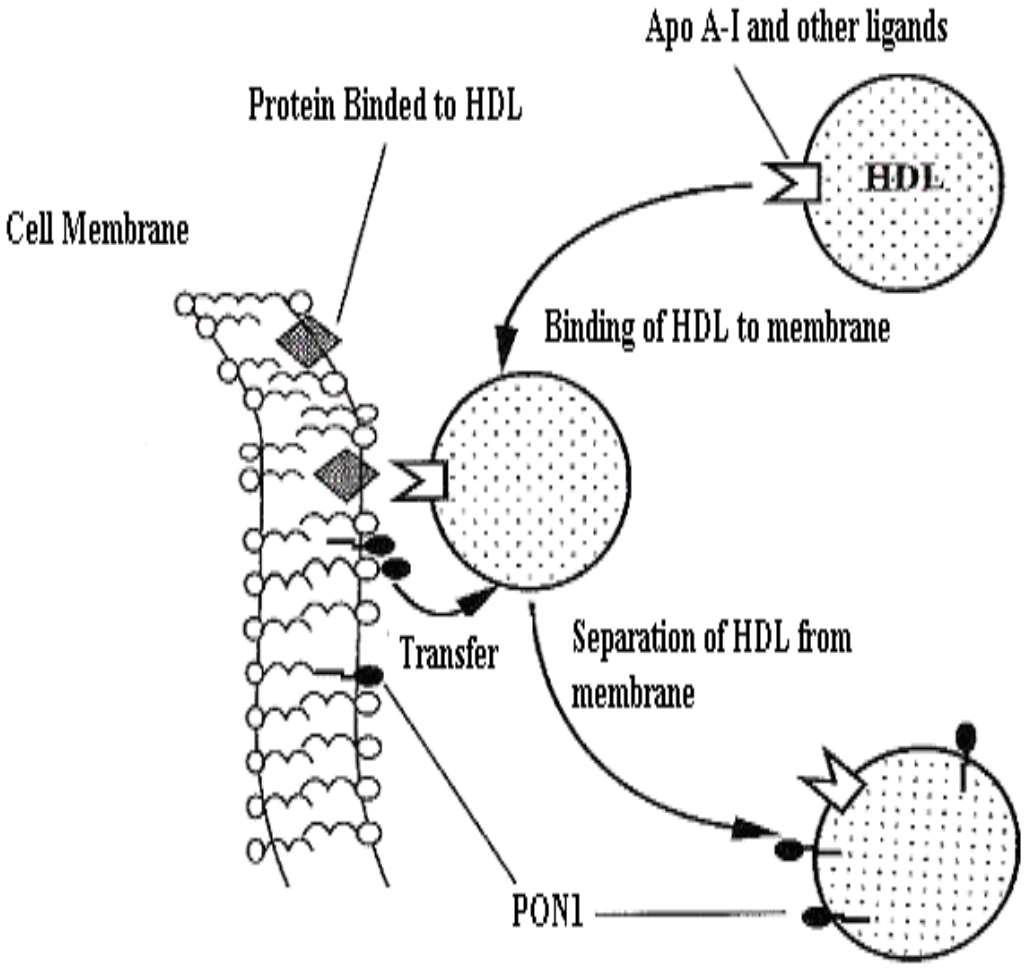

Figure 1.7. Transfer of PON1 from cell to HDL (MacDonald-Wicks et al., 2006)

There are several studies showing that apolipoprotein A1 plays a role in the binding and stabilization of PON1 enzyme to phospholipids, particularly in individuals with high HDL3 fraction and it has been found that PON1 activity is higher in HDL3 with higher Apo J content (Thomas-Moya et al., 2006).

The relationship between circulating the expression and biological role of PON1 could be affected by other proteins related to HDL. For example, PON1 may only interact with the endogenous substrate after it is become free by HDL and may exhibit its biological properties. In contrast, PON1 protects HDL from oxidation. For this reason, an understanding of the relationship between PON1 and HDL is important for the identification of catalytic components (James and Deakin, 2004).

1.5. Lipid Peroxidation

Lipid peroxidation is a chain reaction which provides a continuous source for free radicals initiates further peroxidation. Lipid peroxidation is a chain reaction that provides the destruction of unsaturated fatty acids found in the structure of phospholipids, glycolipid, glycerid and steroids to the products such as alcohol, aldehid, hydroxyl acid, ethane and pentane via oxidants. The membrane damage that occurs in this way is irreversible (Akkuş, 1995; Yerer and Aydoğan, 2000).

One of the most important criteria determining the level of lipid peroxidation, which is one of the important consequences of damage to free tissues of free oxygen groups, is the detection of malondialdehyde (MDA) levels. MDA is not a specific or quantitative indicator of fatty acid oxidation. But it correlates well with the degree of lipid peroxidation. MDA formed as a result of lipid peroxidation can easily identified in blood plasm, and it is used in oxidative stress measurements. In addition, since MDA is soluble in the plasma, it can also be detected in urine (Akkuş, 1995).

Peroxidation of fatty acids containing three or more double bonds leads in the production of malondialdehyde (MDA). MDA, which causes polymerization and cross-linking of the membrane components, changes some properties of inner membrane such as deformability, ion transport and clustering of determinant on the cell surface. Also it can react with nitrogen bases of DNA because it can be diffused.

Due to these properties, MDA is a mutagenic, genotoxic and carcinogenic compound (Akkuş, 1995; Yoneyama et al., 2002).

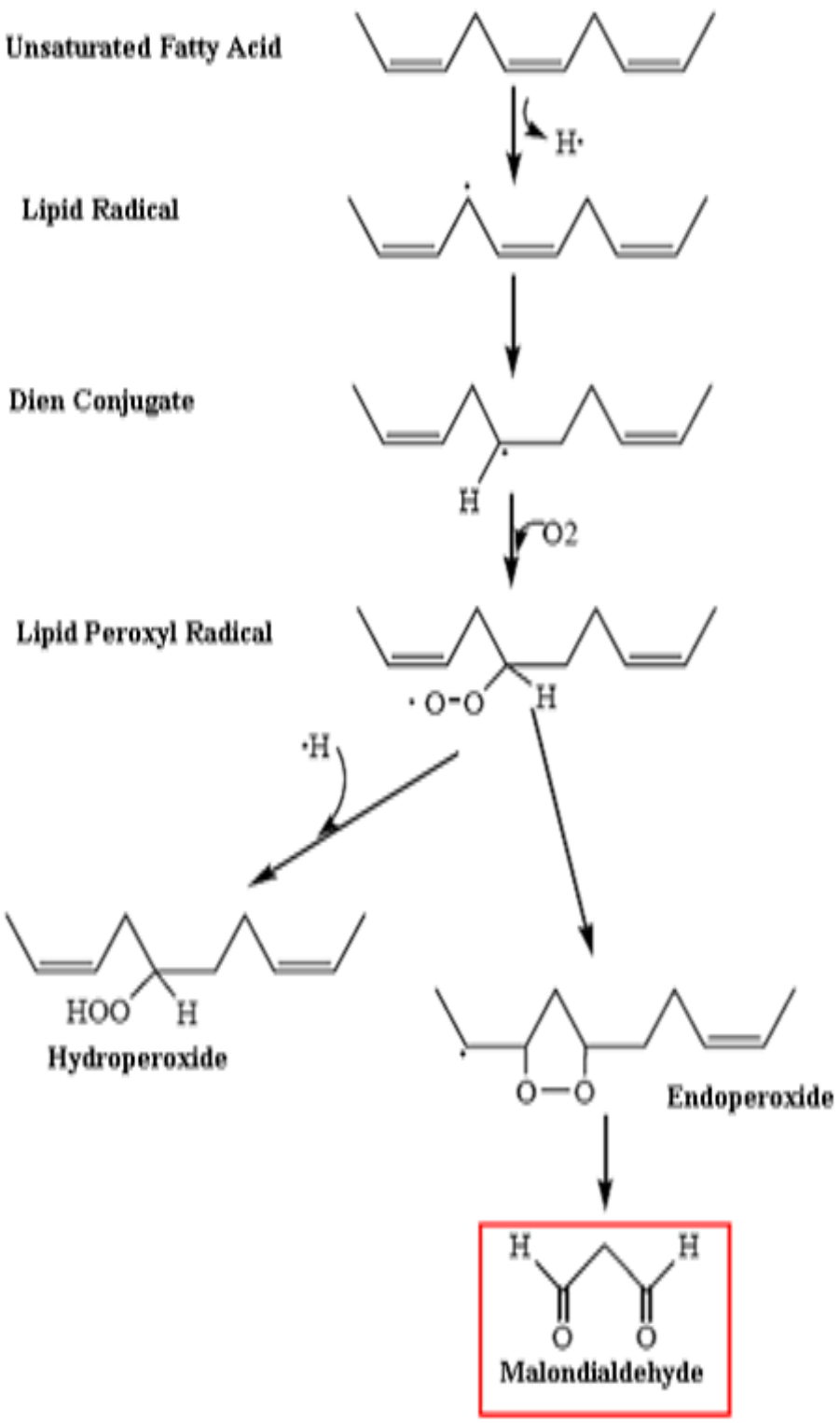

Figure 1.8. Chemical pathway of lipid peroxidation (Murray et al., 1996).

1.5.1. The Results of Lipid Peroxidation in Biological Systems

The products comprise as a result of lipid peroxidation, affect membrane permeability and microviscosity negatively. Oxidation of short chain fatty acids and structural proteins containing amino acids such as tryptophan, tyrosine, phenylalanine, histidine, methionine and cysteine due to peroxidation of fatty acids in the membranes causes membrane permeability to increase and membranous activity to decrease. Lipid hydroperoxides and lipid peroxy radicals show their toxic effects on cellular and metabolic functions react with many components of the same cell, such as free oxygen radicals as follows (Freeman and Crapo, 1982; Köse and Doğan, 1992):

- They cause inactivation of membrane-bound receptors and enzymes.
- They cause the loss of membrane secretion function.
- They break trans-membrane ion gradient. They also increase non-specific permeability to ions such as Ca^{+2}
- They effect oxidative phosphorilation negatively in mitochondria
- They lead to changes in microsomal enzyme activities.
- They cause annihilation of integrity of the subcellular organelles such as lysosomes.

The aim of study investigate the effects could improve the biochemical parameters that accrue in the effective fungicide bellis in *Cyprinus carpio*.In this study, the effect of Bellis, a fungicide commonly used in agricultural production models, on living organisms in aquatic ecosystem was investigated in terms of biochemical values and paraoxonase activity, high density protein and malondialdehyde parameters were selected in order to give an idea on the level of effects.

CHAPTER II

LITERATURE REVIEW

Today, nutrition is one of the most important problems in the growing world population. Food safety is an important event for food producers and consumers, and it raises serious concerns. As a result, the use of pesticides has increased in order to get more yields from agricultural areas. Despite the many benefits of pesticides, there is now serious concern about the use of these substances because of their solubility in water and their toxic properties that harm animals and humans (Karakaya and Boyraz, 1992; Mead et al., 1999; Ezemonye et al., 2009).

The consumption of food is an important pathway for pesticide intake and may cause general public health risk (Liu et al., 2010). Organic pollutants in food accumulate mainly due to their lipophilic and environmental permanence in biological organisms such as fish and meat (Ağca, 2006). Since pesticides such as organochlorine pesticides (OCPs) and Polychlorinated Biphenyls (PCBs) affect human health and the environment, it is important to assess water ecosystems in order to protect human health risks and the environmental ecosystem (Takazawa et al., 2008). When pesticides are used at doses well above the recommended doses, they leave too much residue in the foods (Karakaya and Boyraz, 1992).

The pesticide residues in the foods should not harm people, animals and the environment. Therefore, knowing the amount of pesticide residues is very important for human health and export food products. The tolerance limit of each new-produced pesticide should be determined by formacological and toxicological examinations before being applied to the market (Kınık and Kavas, 2002).

Developing countries prefer to use cheap chemicals like DDT, HCH, BCH. Therefore, environmental contamination, exposure of the public, and food remain at a higher level. The use of these pesticides poses a risk to public health at the same time (Carvalho, 2006).

Although there is little pesticide consumption in our country, unconscious and dangerous pesticide applications are quite common (Bulut and Tamer, 1996). In order to determine possible effects on ecosystem and human health, organochlorine pesticides residues in foods consumed by people such as fish, mussels and milk have started to be analyzed after 1980's (Yatağan, 2008).

Pesticide residues entering by the ways mentioned above are also entering to an organism living in the sea or water (Kaya, 2007). Pesticide residues entered an organism in this way, accumulate in the animal tissue and enter from one organism to another via the food chain (Barlas et al., 2000; Harvey et al., 2008). The biggest depositors of these organic pollutants are the oceans and seas. Persistent organic pollutants (POPs) are stored in sediments in the sea bottom and are transferred through water and the food chain in the atmosphere. A number of studies show that all underground and surface waters in the world are contaminating with pesticides and their derivatives. Although the use of DDT and Beta-hexachlorocyclohexane (BHC) has been terminated in the last decade in the world, these pesticides are found above the permissible values, especially in developing countries. Pesticide and its derivatives transmitted from household, industrial and agricultural wastes are also transferred to food chain through surface and underground waters (Hongsheng, 2011; Bansal, 2012).

Qualitative and quantitative changes happen within bioavailability and bioaccumulation depend on various biotic (habitat, food chain status, detoxification mechanisms, sexual maturity/physical factors, gender, fat content, nutritional pattern, tissue composition and metabolic capacity) and abiotic (pysical and chemical properties of chemicals, environmental factors such as light, dissolved oxygen concentration, salinity, pH, and temperature). While these factors alone are not enough, there may be differences in residual levels depending on what species and contaminants are (Ozçelik et al., 2011).

The distribution and transportation of the pesticides in water depends on both their chemical structure and formulations in addition to environmental conditions. Some pesticides are soluble in water and homogeneously dispersible, while some inorganic salts are precipitated in water. The absorption of the pesticides by organisms living in the water depends on pesticide level in the water, the physiology of the organism, the

temperature and pesticide residues existing previously in the structure of the organism (Atamanalp, 2004; Mahmoud and Loutfy, 2012).

Pesticide residue concentrations are 1000-10000 times more in fish than in water. Although consumption of fish and aquatic products is very beneficial for human health, these products are an important source of human intake of these chemicals. In this context, fish is considered as an indicator because it can contain more than these substances. The amount of pesticide residues varies according to the species and size of the fish (Barlas et al., 2000; Aktümsek et al., 2002; Storelli et al., 2004). Consuming foods contaminated with pesticides is a very dominant way to get these things into the body. In some studies it has been reported that there is a significant correlation between organochlorine concentrations in humans and fish consumption (Raeside, 2007).

Rico et al. (1987) investigated the degree of organo-chlorine contamination in the common carp (*Cyprinus carpio*) sampled from the main water supply of the Danone National Park in Spain. As a result of the study, 0.06 ppm DDT, 0.19-0.35 ppm PCBs, 0.02-0.07 ppm DDE and heptachlor, heptachlor epoxide and dieldrin pesticides in low amounts have been found in *C.carpio*.

Westernhagen (1988) reported an increase in total protein, structural protein and soluble protein, and a decrease in amino acid and protease activity in *C.carpio* exposed to sublethal doses of malathionin for 7-15 days. In case of expulsion of pesticide to 30 days, it has been reported that the stated values are close to normal.

Costa et al. (1990) showed that PON1 enzyme, purified from rabbit serum and injected intravenously into rats, increased serum PON1 activity 9 times against paraoxon and 50 times against chlorpriphosoxone. After exposure of these rats to any of the organic phosphorus compounds, the level of acetylcholinesterase inhibition in different tissues was measured and it was observed that the injected PON1 increased the resistance of rats to chlorpriphosoxide, in particular. It has been reported that this protection is more pronounced in brain and diaphragm tissues and is also effective when contact with organophosphate compounds occurs via the skin.

Kaur and Dhawan (1993) found that 10 mg / L of phosphamidon did not have any effect on the viability of the *C.carpio* larvae, whereas 0.1 mg / L of carbofuran and Malathion and 1 mg / L doses have a negative impact on viability.

The effects of synthetic organic compounds, which are most widely used today, on aquatic products, have been the subject of numerous researches. According to Weber (1977), organic chlorinated pesticides are more toxic than organic phosphorous pesticides and organic phosphorous pesticides are more toxic than organic acids, according to a study done with rainbow trout (Canyurt, 1994).

In a study conducted in Abou-Arab et al. (1996), 1,12-bis (4-chlorophenyl) ethane (p, p'-DDA), lindane, endrin, heptachlor and malathion pesticides are found in high amounts in sardine and mackerel fish collected from Egypt; Aldrin, -benzenehexachlor (-BHC) and methyl parathion were found to be low.

Philip and Rajasree (1996) have reported that the sublethal dose of Cypermethrin causes increment in soluble protein and total protein, free amino acid, and protease activity, transaminase, aspartate aminotransferase, alanine aminotransferase and glutamate dehydrogenase levels.

Ayas et al. (1997) found 13 different organochlorine pesticide residues in a study of some animals in the Göksu Delta. Heptachlor, heptachlor epoxide and DDT were detected in the liver and adipose tissues of carps caught from Göksu Delta, while BHC, lindane, aldrin, dieldrin, endrin pesticides remained uncovered. However, they found that organochlorice (OC) pesticide residues accumulate more in the fat of fish than in the liver.

Oruç and Uner (2000) examined the hepatic antioxidant enzyme activities and lipid peroxidation levels in *O.niloticus* by applyin azinphos methl (27 ppm 2,4-D+0.003) for 24, 48, 72 and 96 hours period. They found that there is a high increase in glucose-6-phosphate dehydrogenase (G6PD) and glutathione reductase (GR) activities and there is not any change in the level of MDA compared to the control group.

In a study investigating the detection of organochlorine pesticide residues in sea bass fish on the Lake Beyşehir, 85% of the fish were found to be contaminating. In the

samples, aldrin, dieldrin and endrin are found in small amounts and heptachlorin is found in only one sample (Aktümsek et al., 2002).

Li et al. (2003) reported that the amount of MDA in the liver of *Carassius auratus*, which has been exposed to 3,4 dichloroaniline for 15 days increased, on the other hand the amount of GSH decreased.

Sevgiler et al. (2004) reported an increase in the amount of MDA in the liver of the *Oreochromis niloticus*, which was exposed to different concentration of etaxazole for 1, 7 and 15 days, on the seventh day.

Increasing numbers of toxic organic chemicals in the aquatic environment may have a detrimental effect on the health of populations. It is therefore of utmost importance to determine the relationship between the level of pollution and their biological effects before any harmful effect occurs at higher organizational levels. In this context, studies on the consideration of pesticides and other chemicals play an important role in the assessment in organisms in aquatic organisms (Boyuneğmez, 2004).

Lushchak et al. (2005) reported that the amount of oxidative damage products in the study of brain, liver, kidney and muscle tissues in *C. carpio* was reduced in other tissues except liver tissue, usually after five hours, to increase in liver tissue in order to investigate hypoxia and reoxygenation effects. It was observed that the amount of carbonyl proteins was highest in the kidney, lowest muscle area under normal conditions, and the amount of PCO in brain tissue alone was not affected during hypoxia and aerobic recovery. In the case of aerobic healing, the amount of PCO increased by 50-58% compared to hypoxic and control groups at 14 h in the liver. When muscle and kidney tissues were compared with control, decrease was observed in both groups. The amount of lipid peroxides is highest in the control fish and lowest in the brain and muscle. During hypoxia it has been observed that LPO decreases in brain and liver tissues, and there are no significant changes in kidney and muscle. While glutathione does not change in all tissues under hypoxic conditions, it has nearly doubled in liver and kidney tissue at the end of 14 hours of aerobic recovery.

Durmaz et al. (2005) investigated the effects of diazonin on antioxidant enzyme activities of various tissues of *O.niloticus* in their study. They found that diazonin caused an increment in MDA levels.

In 4 species of fish (*Acanthobrama marmid*, *Cyprinus carpio*), *Chondrostoma regium* and *Silurus glanis*) consumed by humans and different nutritional behaviors consumed by people in Kahramanmaraş, the residue levels of pesticides, polychlorinated biphenyls and polybrominated biphenyl ethers were examined. DDTs were the predominant pollutants in all fish species. In particular, p, p'-DDE is measured at around 90% of the total DDT. OCP concentrations in the medium are higher than other species, depending on the biscuit (fish-fed) diet and high fat content. In Karaburun fish, depending on the herbivor feeding style, levels are lower than other species. It is also seen that OCPs accumulate in the muscle and muscle in the spring and in the carapace, and in pitfalls, p, p'-DDT accumulate in the liver deliberately higher in the liver (Erdogrul et al., 2005).

Durmaz et al. (2006) found that no significant changes in MDA levels in the gill region of *O.niloticus* exposed to sublethal dose of diazinon for 1, 7, 15 and 30 days is seen. On the other hand they also found that the amount of MDA levels increased witj long-term applications and higher doses.

Yılmaz et al. (2006) examined the effects of pollution in Karakaya Dam Lake (Malatya) on *Cyprinus carpio* liver catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities. They reported that there is an increment in CAT activity and a decrement in SOD and GSH-Px activities.

Dongyun et al. (2006) investigated the quantitative structure and toxic effecs of Ops in *C.carpio*. As a result of the study they found that, important mechanisms can be clarifed by modelling chemical reactivity in the homologous series of organophosphorus compounds related to toxicity at different levels in *C.carpio*.

Oruç and Usta (2007) investigated the effects of diazonon on antioxidant enzyme activities in different tissiues of *C.carpio*. As a result of the study they reporte that diazonon caused and increment in MDA levels.

Li et al. (2007) reported that the amount of GSM in the liver of *C.auratus* exposed to different concentrations of 2,4,6-trichlorophenol decreased, MDA level significantly increased.

Sediment, mussels and water samples were collected 3 times between 2001 and 2003 from 9 stations along the Coast of Blach Sea in order to investigate the distribution and accumulation levels of OCPs. The levels of DDT and metabolic products are well above the limits. The highest measured DDT metabolite in sediment was 35.9 ng/g and the median was 14.0 ng/g wet weights. In addition, the samples contain considerable levels of aldrin, dieldrin, endrin, heptachlor epoxide, lindane, endosulfan sulfate and HCB (Ozkoç et al., 2007).

Işık and Celik (2008) reported that while fluctuations seen in GSH amoun compare to the control group, MDA levels increased in *O. myciss*, exposed to methyl parathion and diazinon at 24, 48 and 72 hours.

Franco et al. (2008) investigated biochemical changes in *C.carpio* that are targeted to glutathione reductase and exposed to zinc. As a result of the study they found that glutathione reductase obtained from *C.carpio* is inhibited and zinc toxicity has an important mechanism.

Beketov and Liess (2008) investigated the effects of different pesticides (thiacloprid, imidacloprid, acetamiprid, iprodione, fenvalerate, and indoxacarb) on *Simulium latigonium* larvae and *Gammarus pulex*. As a result of the study it has been found that a 96 hour LC50 value of 2520 μg / L for Indoxacarb *Gammarus pulex* and found that the amount of indoxacarb residue was significantly higher than the LC_{50} value for live exposed to indoxacarb sublethal concentration for 4 hours.

Dinçel et al. (2009) reported that there brain MDA levels increased after 1 week; on the other hand there was no significant difference in the amount of MDA in between control and cyfluthrin groups in the liver tissue of *C.carpio* exposed to sublethal dose of cyfluthrin.

Sepici et al. (2009) have exposed *Cyprinus carpio* for 48 hours to a sublethal dose of Cyfluthrin (10μg/L). As a result of the study, it has been determined that MDA levels in brain tissue have increased.

Korkmaz et al. (2009) reported that the amount of protein in the liver tissue of O. niloticus, which was released by cypermethrin, decreased compared to control groups.

Moreno et al. (2010) reported that the amount of MDA in brain tissue of Tinca tinca was greater than that of control, under the influence of different concentrations of carbofuran and deltamethrin.

Düzgüner and Erdoğan (2010) reported that 10 μM imidaclopridine cause an increment in MDA levels in plasma and liver tissues of rats administered intravenously and there was no significant difference in brain tissue compared to control groups.

El-Gendy et al. (2010) reported that the amount of MDA increased significantly and the amount of GSH decreased when imidacloprid-treated rats were compared with controls at the liver.

Jin et al. (2010) reported that the amount of MDA in the adult female Danio rerio liver affected by different concentrations of atrazine was higher than the control.

Suvetha et al. (2010) examined the effects of cypermethrin toxicity on ionic regulation and sodium-potassium activity in *Cyprinus carpio*. As a result of the study the reported that Na^+K^+ATPase plays an important role in osmotic and ionic balance in bronchial ion transport. A decrease in Na^+K^+ATPase activity was also detected.

Moraes et al. (2011) examined toxic responses in *C.carpio* exposed to commercially available imazethapyr and imazapic. They reported that herbicides in fish metabolism cause toxic effect in long-term.

Ochoa-Acuna et al. (2009) suggest that the adverse effects of conazole fungicides in nontarget species may be mediated through cytochrome P450 pathways common across species.

Strong evidence in support of such expectations is provided by Mazur and Kenneke (2008) who report similar, and in some cases identical, in vitro metabolite profiles for conazoles in trout, rat, and human liver. Other examples are provided by chlorothalonil, which exerts its toxic effects on fungi by complexing with

sulphydryl-containing proteins, leading to depletion of glutathione reserves (Arvanites and Boerth, 2001); some of these same thiol-reactive processes are affected in fish (Davies et al., 1994) and invertebrates (Davies et al., 1994; Baier-Anderson and Anderson, 2000). Azoxystrobin affects respiration in fungi by inhibiting electron transport in mitochondria, leading to cellular oxidative stress and disruption of fungal metabolism and growth.

Recent studies indicate that azoxystrobin disrupts mitochondrial respiration in both fungi (Gisi and Sierotzki, 2008) and fish (Olsvik et al., 2010). Imidazoles, triazoles, and the pyrimidine fungicide fenarimol belong to the cytochrome P450-de-methylase inhibiting (DMI) class of fungicides, but disrupt other CYP450s, such as aromatise (CYP19) in both mammals and fish, indicating endocrine disruptive action is associated with DMI fungicides (Sisman and Turkez, 2010). While such biochemical insights do not allow cross-species predictions of toxic potency, they do provide a first step towards identifying potential MOAs in aquatic invertebrates and fish for which mechanistic studies of fungicide action have not been conducted.

Although paraoxonase (PON) was being perceived as two separate enzymes such as aryldialkylphosphatase (EC3.1.8.1) and arylesterase [(ARE) EC3.1.1.2], but the studies performed revealed that they had both ARE and PON activity was encoded by the single gene (Mackness et al., 1998). Enzyme was first detected in human serum after electrophoresis in 1961 in HDL immuno-precipitates. Then it has been shown that purified PON is associated with lipids and the molecular weight is the same as HDL.

In 1988, Mackness and colleagues found that PON activity was dependent on apo AI on HDL, and in 1991 also found that PON decreased peroxide accumulation on LDL. Population studies have shown that there is a statistical relationship between enzyme activity and HDL, apo AI, apo AII (Azarsız and Sözmen, 2000).

Serum PON1 is found in plasma with HDL and is thought to play a role in the prevention of plasma lipoprotein oxidation. Mice lacking the PON1 enzyme become sensitive to diet-induced atherosclerosis. The fact that this enzyme is always associated with HDL in plasma has a significant contribution to the antiatherogenic effects of HDL. As peroxidized lipids are metabolized by this enzyme, accumulation

of lipid peroxides in both HDL and LDL is prevented. Because of this property, PON1 is responsible for the protective effect of HDL against oxidation of LDL and is therefore more effective than vitamins A and E (Rousselot et al., 1999).

There are many studies on whether PON1 activity is related to the HDL cholesterol and apolipoprotein AI concentrations. PON1 activity is significantly reduced in Tangier disease and Fish eye disease with HDL deficiency, while there is no significant decrease in other HDL deficiencies (James et al., 1998).

The best known of the various aldehydes resulting from lipid peroxidation are malondialdehyde (MDA) and 4-hydroxynonenal (HNE). These compounds either metabolize cellularly or diffuse to the area where they are effective at the onset, releasing the damage to other parts of the cell (Nair et al., 1986; Halliwell and Chirico, 1993). Lipid peroxidation can be assessed by MDA measurement.

In a study conducted by Sevgiler et al. (2004) it has been found that etaxosole in the liver of freshwater fish *Oreochromis niloticus* caused increment in MDA levels.

Eraslan et al. (2007) found that CAT and SOD activities were decreased in erythrocytes and MDA levels were increased in the plasma of mice given Cyfluthrin.

In a study conducted by Düzgüner and Erdoğan (2010) to investigate the effects of imidacloprid on acute oxidant and inflammation in the nervous system and liver of rats, it has found that the amount of MDA in plasma and liver was increased compared to control, the change was not in brain, CAT activity in both tissues did not change, Three times more in the brain than in the liver.

In a study to investigate the possible effects of c-synthetic pirytroid Lamda-Cyhlothrin (LTC) conducted by Fetoui et al. (2009), it has been found that while MDA levels increase, vitamin C reduce this increment.

Deveci (2012) investigated the protective effect of CAPE on PON1 activitiy and HDL levels in the plasma and brain tissue of mice with experimental Parkinson used chlorpyrifos ethyl (CPF). Deveci found that PON1 and HDL levels were significantly lower in CPF group.

CHAPTER III

MATERIALS AND METHOD

3.1. Materials

This study was conducted on one species (*Cyprinus carpio*) belongs to *Cyprinidae* family. A total of 24 fishes (200-300 g) obtained from Islahiye Tahtaköprü. The fishes were transported to the laboratory in polyethylene thanks for a period of 5 days (22-25°C and normal photoperiod). The new environment has provided adaptation for them. Then they were divided into 3 groups of 24 fish: A control group contain 8 fish, group of 0.025 mg/ L Bellis I contain 8 fish and group of 0.050 mg/L Bellis II contain 8 fish. After the end of 14 days of application, blood samples were taken from the fish and the plasma were separated. Paraoxonase (PON1) activity, High Density Lipoprotein (HDL) and Malondialdehyde (MDA), were analyzed in the obtained plasma samples.

In this study we constituted 3 groups;

1. Control Group: This group includes the *Cyprinus carpio* without any fungicide

2. Bellis I: This group includes the *Cyprinus carpio* with 0.025 mg/L Bellis

3. Bellis II: This group includes the *Cyprinus carpio* with 0.050 mg/L Bellis

3.1.1. Taking and Processing of Blood Samples from Animals

Twenty four hours after the last administration, blood samples were taken caudal vena into the heparinized tubes. Blood samples were centrifuged at 3000 rpm for 15 minutes and plasmas were obtained. The samples were stored in deep freeze at -20°C.

3.2. Method

3.2.1. Tools and Materials Used in the Study

1. Spectrophotometer (Microplate Reader Epoch, Shimadzu UV-1201)
2. Refrigerator (-20°C) (Bosch)
3. Water bath (SB 100, Nuve)
4. Sensitive scales (Scaltec)
5. PH meter (inoLab)
6. Vortex (Labinco)
7. Centrifuge (Helius, Germany)
8. Adjustable automatic pipettes (5-10 µl, 10-100 µl, 100-1000 µl; Varipette 4710, Germany)

3.2.2. Consumables Used in the Study

1. Butulated hidroxy toluen (BHT) (Merck)
2. TBA (Sigma)
3. TCA (Merck)
4. NaOH (Merck)
5. Na_2HPO_4 (Merck)
6. Paraoxon (Supelco)
7. Calcium chloride (Sigma-Aldrich)
8. Tris-HCl (Sigma-Aldrich)
9. Hydrochloric acid (Merck)
10. Acetone (Sigma-Aldrich).

3.2.3. Biochemical Analyses

3.2.3.1. Plasma Paraoxonase Activity (PON1) Analysis

Paraoxonase activity was analyzed according to methods of Eckerson et al. (1983) and Gülcü and Gürsu (2003).

Principle:

It is based on the measurement of the absorbance of p-nitrophenol comprised as a result of the hydrolize of paraoxon (0,0-dietil-0-p-nitrofenilfosfat) by PON1 at 412 nm spectrophotometrically at 25°C.

PON1 activity was calculated as U/L, taking into account the molar absorption coefficient determined for p-nitrophenol and the dilutions made in the experiment. Enzyme activity which converts 1 μmol paraoxon into para-nitrophenol in 1 minute for paraoxonase activity is defined as Unit (U) (Eckerson et al., 1983).

Solutions used for PON1 Measurement:

a. **HCL Solution (0.1 M) (a):** The volume of 10 ml, 1 M HCl solution was completed to 100 ml with distilled water.

b. **Tris Solution (0.1 M) (b):** 1.21 g tris soluted in distiled water and the total volume was completed to 100 ml

c. **Tris-HCI Buffer (20 mM, pH: 8):** 29 ml "a" solution (HCL solution) and 50 ml "b" solutions (Tris Solution) was mixed; the pH of new mixture was adjusted to 8. The volume of the mixture with pH 8 was completed to 100 ml with distilled water.

d. **Study Reagent [calcium chloride (2 mM) -paraoxon (2 mM)]:**29.4 mg calcium chloride (CaCl2.2H2O) was dissolved in a little tris-HCL buffer. The volume was completed to 100 ml adding tris HCl buffer by 44 μL paraoxon dissolved in 1.5 mL acetone.

e. **Acetone:** It was taken from the acetone bottle with normal usual purity.

Paraoxonase Analysis:

280 µl of working reagent is added in sample and blind wells. Then 8 µl of distilled water is added to the blind plug and 8 µl of plasma is added to the sample well. The absorbance increase of the sample is measured at 412 nm against blind for 2 minutes at 25°C (ΔA/min: Difference in absorbance per minute).

$$\text{U/L (µmol/min/L)} = \frac{\Delta A/\text{min} \times SF \times 106}{\varepsilon \times 1/0.6}$$

ΔA/min : Change of absorbance in a minute

E : The molar absorption coefficient of p-nitrophenol, 18290 for the current test conditions

SF : Dilution factor (Total volume / Sample volume)

106 : µmole conversion factor

1/0.6 : The length light path

3.2.3.2. Plasma High Density Lipoprotein (HDL) Analysis

Plasma HDL level was analyzed with an automated analyzer (Huma Star 600, Germany) using a commercial kit.

3.2.3.3. Plasma Malondialdehide (MDA) Analysis

To determine lipid peroxidation (MDA) in plasma, a spectrophotometric method as described by Ohkawa et al. (1979) was used.

Principle

Incubation of the plasma with thiobarbituric acid (TBA) at 100°C in an aerobic medium with a pH of 3.4, produces malondialdehyde (MDA), a secondary product of lipid peroxidation. The resulting MDA makes a pink complex with TBA. Lipid

peroxidation is detected by spectrophotometric measurement of the pink color at 532 nm.

10µl of 1,1,3,3 tetraethoxypropane were takein for curve drawing. It was then dissolved in 10 ml of absolute ethanol and stored in a dark glass at +4ºC. Study solutions with different concentrations were prepared from this stock solution and a standard curve was drawn. The determined absorbance values were calculated as nmol/ml from the MDA standard curve.

Solutions Used for MDA Measurement:

a. Serum Physiologic Bufferd with Phospahate Buffer (pH: 7.4):

- 8.1 g NaCl
- 2.302 g Na2HPO4
- 1000 ml distilled water

b. BHT Solution (0.88%):

- 88 mg of BHT are weighed and dissolved in 10 ml of absolute alcohol.

c. TCA Solution (30%):

- 30 g TCA is completed with distilled water to 100 ml.

d. EDTA Solution (0.1 M):

- 37.224 g of EDTA-Na2 2H2O are weighed and completed 1 liter with distilled water.

e. TBA Solution (1%):

- 1 gr TBA
- 100 ml 0.05 N NaOH

f. NaOH Solution (0.05 N):

- 2 g NaOH is completed 1 liter with distilled water

Malondialdehide Analysis:

0.8 ml phosphate buffer, 0.025 ml BHT and 0.5 ml 30% TCA were added to 0.2 ml sample. The tubes were mixed with vortex and allowed to stand on ice for two hours. It was then centrifuged at 2000 rpm for 15 minutes. One ml of supernatant was transferred to another tube. 0.075 ml of 0.1 M EDTA and 0.25 ml of 1% TBA were added. After the tubes were mixed and allowed to stand for 15 minutes in the water bath, the absorbance was read at 532 nm in the spectrophotometer. BHT, an antioxidant, was used to prevent MDA formation which could result in erroneous high TBA reactivity during measurement.

3.2.4. Statistical Analyses

Statistical analysis of the data obtained from the study was carried out with SPSS (Statistical Package for Social Sciences) package program. One-way analysis of variance (ANOVA) was used to determine whether there is a difference between the means of the test groups and the "Anova-Duncan" test was applied to the group averages to determine the group or groups from which this difference is observed if there is a difference between the averages of the test groups. The results were considered at 95% ($p<0.05$) significance level.

CHAPTER IV

RESULTS

In the 18th century, after the industrial revolution, agricultural production had to be increased at the same time in order to meet the rapidly growing population nutrition needs. Therefore, industrial farming model has started to be widely applied in the world with the use of plant protectors, plant growers, artificial fertilizers and drugs developed against plant pests instead of the agricultural model which is traditionally made with low efficiency from the unit side. This production model increased the level of crop production and the yield from the unit area. Later, however, it began to focus on the issue of which all chemicals and infestations used could harm the ecosystem with the studies conducted by scientists. It has been understood that the studies carried out as to how the chemicals and pesticides used lead to the increase of the production in the plant and the changes which are caused in the plant, and that the areas where these preparations are used are a dynamic area and that they can also interfere with the soil and aquatic ecosystem. In addition to biochemical tests, molecular tests have been started as an area that has developed in recent years in studies investigating the on-the-live effect of harmful pests preventing plant growth. Fungicides used against fungi, which may adversely affect the level of agricultural production, are particularly flow in to water resources. Fungicides show this feature by preventing sport germination, germination tube and micelle development and sporulation. The answer to this question of how fungi in water resources can be affected by this, the extent to which the level of efficacy of fungicide-bearing preparations may be toxic, is crucial for the sustainability of aquatic life and food safety for those who ultimately consume them. For this purpose, we have selected fish (*Cyprinus carpio*) which is an important indicator in the aquatic ecosystem and showing similarity to human in terms of detoxification mechanisms in order to show the level of toxicity by biochemical parameters in our experimental study (Figure 4.1). In our study, three groups were formed: the control group, 0.025 mg/L Bellis I group and 0.050 mg/L Bellis II group. Blood samples were taken from

the fish at the end of 14 days of application and the plasmas were separated. Paraoxonase activity, high-density lipoprotein and malondialdehyde levels were analyzed in the obtained plasma samples (Table 4.1).

Figure 4.1. Carps used in study (Original).

Tables and graphs of the result obtained from the experiment are given below.

Table 4. 1. Comparision of PON1, HDL and MDA levels between the groups.

PARAMETERS	GROUPS			
	Control (n = 8)	Bellis I (0.025 mg/L) (n = 8)	Bellis II (0.050 mg/L) (n = 8)	p<
PON 1 (U/L)	29.86±3.66[a]	20.07±2.36[b]	18.55±1.96[b]	*
HDL (mg/dl)	36.42±2.44[a]	29.57±2.44[b]	26.85±2.99[b]	*
MDA (umol/l)	1.47±0.13[a]	1.74±0.25[b]	1.83±0.24[b]	**

[a, b] There are significant differences between the averages with different letters in the same row (*: $p<0.001$, **: $p< 0.05$). There is not significant difference between the avarages with same letters in the same row ($p>0.05$)

4.1. Malondialdehide (MDA) Result

Statistically significant difference was found between control and Bellis I groups in terms of MDA amount ($p<0.05$). In addition significant difference was found between control and Bellis II groups ($p<0.05$). As a result of the statistical analysis it was determined that the difference between Bellis I and Bellis II was not significant ($p>0.05$) (Table 4.1).

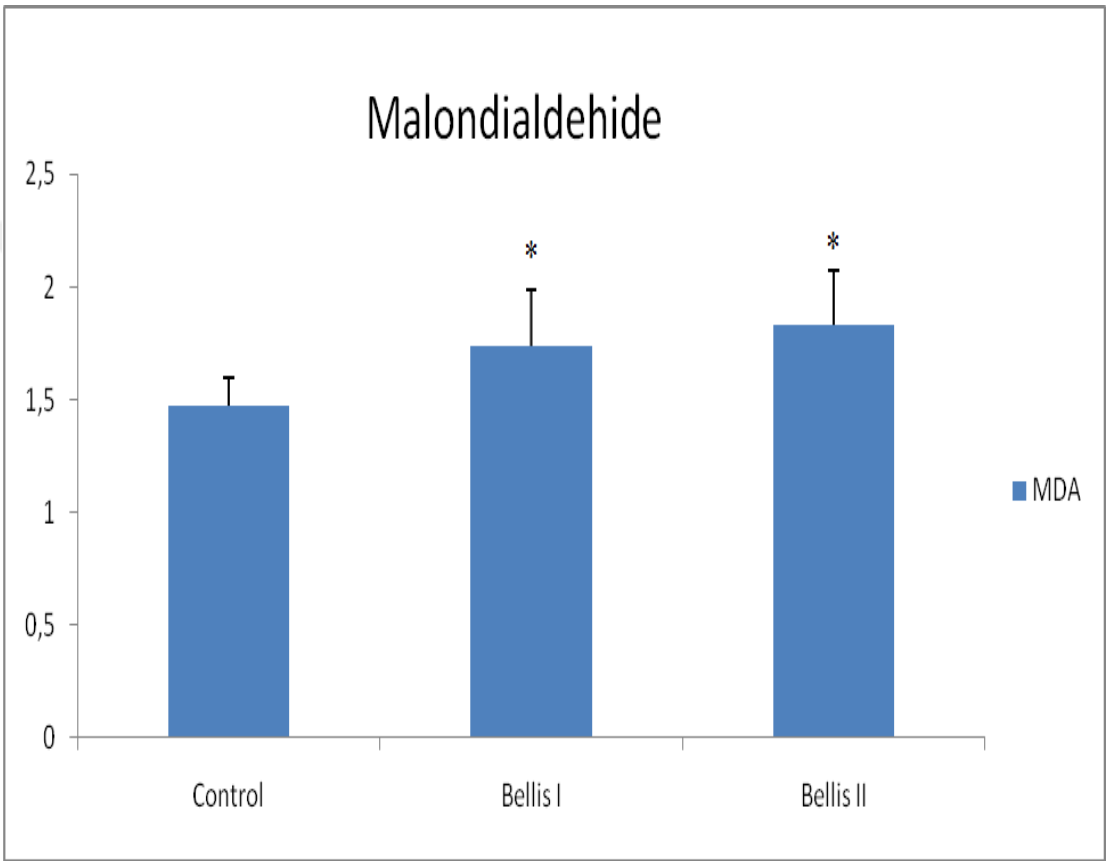

Figure 4.2. Malondialdehyde levels of all three groups. ***:** Significantly differre from control group at $p<0.05$.

4.2. High Density Lipoprotein (HDL) Result

In terms of biochemical parameters, HDL levels were higher in control group than other groups. The difference between the control group and both Bellis groups was statistically significant ($p<0.001$). Although HDL levels of Bellis II groups were lower than Bellis I, no statistical difference observed between this groups ($p>0.05$).

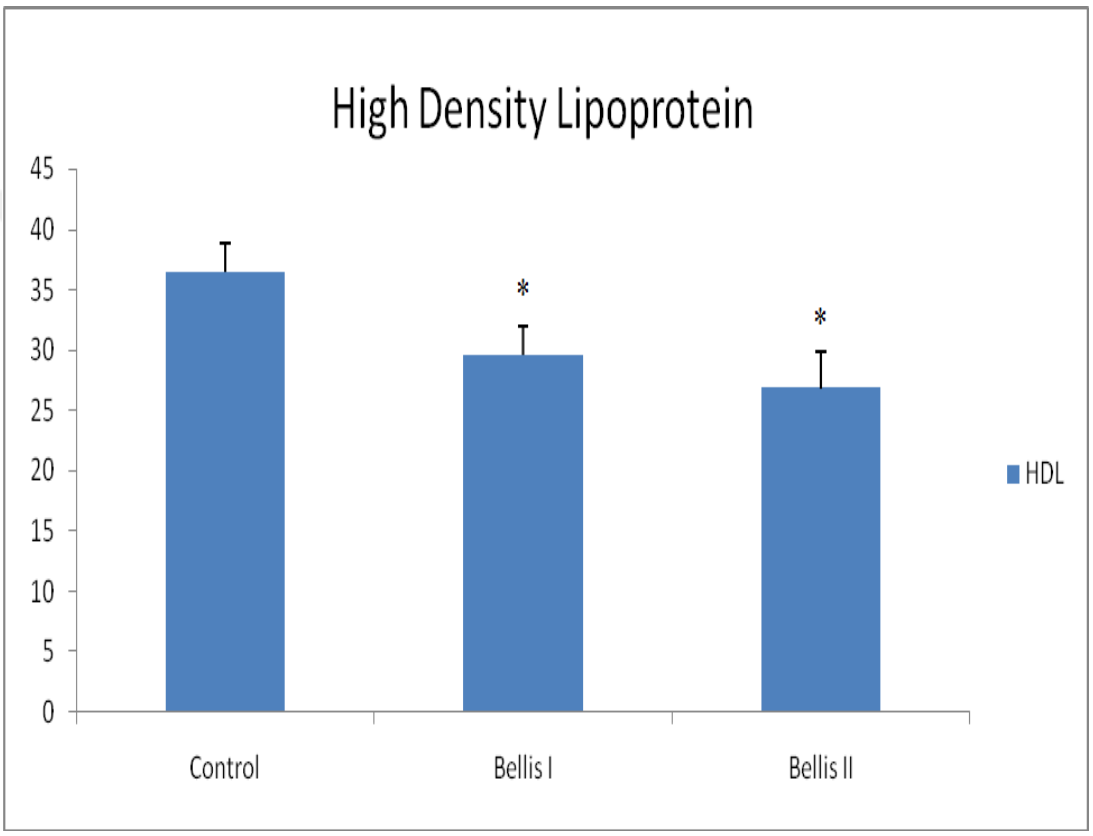

Figure 4.3. High Density Lipoprotein levels of all three groups. ***:**Significantly differre from control group at $p<0.001$.

4.3. Paraoxonase Enzyme (PON1) Result

In terms of biochemical parameters, PON1 levels were higher in control group than other groups. The difference between the control group and both Bellis groups was statistically significant ($p<0.001$). Although PON1 levels of Bellis II groups were lower than Bellis I, no statistical difference observed between these groups ($p>0.05$).

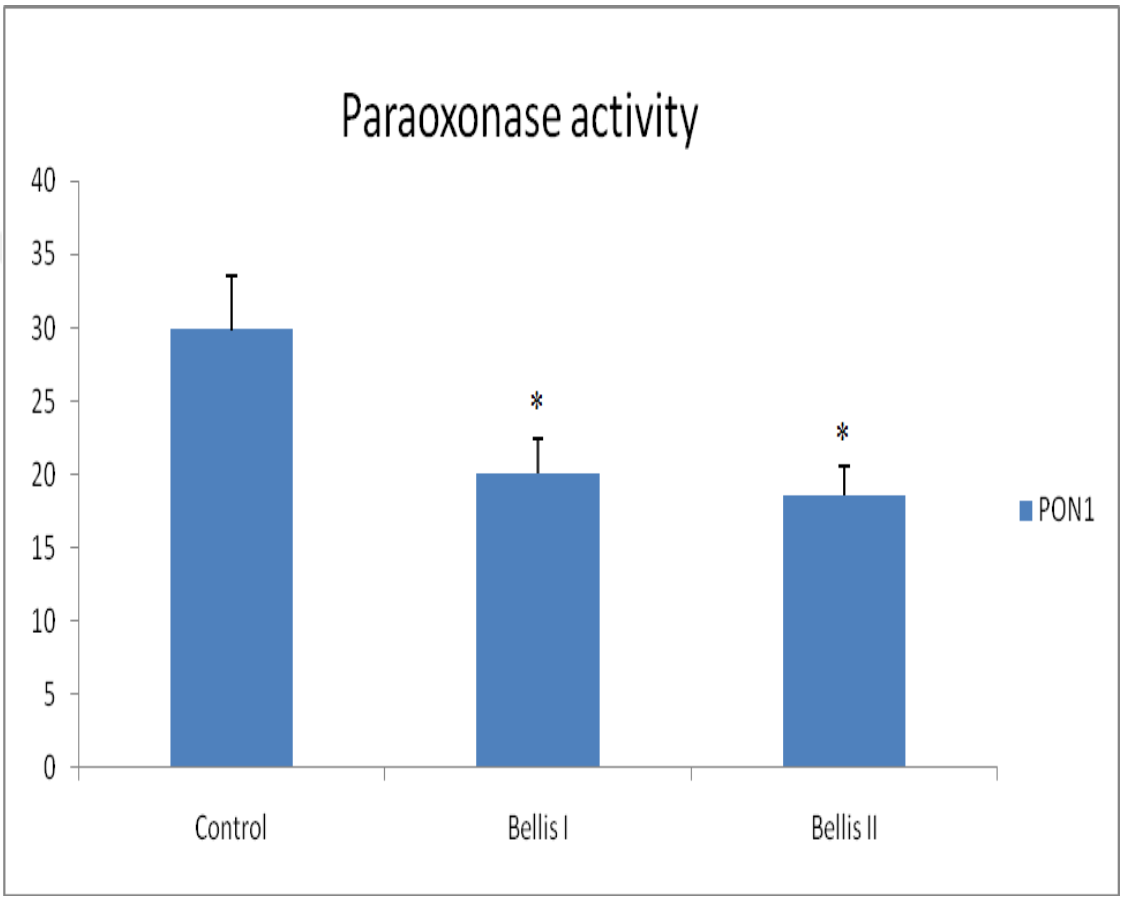

Figure 4.4. Paraoxonase activities of all three groups. ***:**Significantly differre from control group at $p<0.001$.

In this study, the effect of Bellis, a fungicide commonly used in agricultural production models, on living organisms in aquatic ecosystem was investigated in terms of biochemical values and paraoxonase activity, high density protein and malondialdehyde parameters were selected in order to give an idea on the level of effects. As a result of the experimental edictions and analyzes, it is understood that when the Bellis is mixed into the aquatic ecosystem, the antioxidant system may be

adversely affected on the living organisms, whereas the oxidant stress is increased due to the oxidative stress. Analyzes show that these changes in the resulting biochemical parameters make a significant difference between the control and the other two Bellis groups (Bellis I, Bellis II) ($p < 0.001$, $p < 0.05$). No significant statistical difference was found between Bellis I and Bellis II groups in terms of paraoxonase activity, high density lipoprotein and malondialdehyde levels ($p > 0.05$).

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CHAPTER V
DISCUSSION AND CONCLUSION

Although pesticides used to obtain more crops and to protect food from harm, these chemicals have negative effects on the ecological system and live organisms. Pesticides have begun to be preferred as they make it easier to control the harmful effects of the some insects on agricultural products. Chlorpyrifos-ethyl, an organophosphorous insecticide nowadays, is a pesticide commonly used in agriculture. Although this insecticide targets bugs that damage plants, it also affects living creatures living in aquatic ecosystems such as rainwater, drainage waters and underground waters, such as rivers, lakes and seas. In addition, the bioconcentration of insecticides in the aquatic ecosystem reaches the highest level in fish through the food chain. Many studies have reported that the acute and chronic effects of pesticides via food chain have genotoxic, embryotoxic, neurotoxic and carcinogenic effects on living organisms (Stephen et al., 1997; Eaton et al., 2008; Deveci, 2015).

Aquatic environment continuously contaminated by the toxic chemicals used in industry, agriculture and domestic activities (Begum, 2004).

Pesticides are taken up by organisms such as epidermis, gill, and digestive system. These chemical substances, taken in each way, go through a variety of biological membranes until they are assimilated in the body. The structure of these biological membranes and toxic compounds are the most important factors controlling entry into the organism. For example, small moleculed substances and substances dissolved in oil are taken by passive diffusion, which does not require energy use. Accumulation of the compounds in the body through passive diffusion varies depending on factorssuch as the metabolic rate of the animal, nutritional status, size, age and temperature of the ambient water.

The distribution systems in the organism (body fluids) play an important role in the transition of matter from the biological membranes to the organs. Concepts such as bioavailability, bioconcentration, bioaccumulation and biomagnification are used when expressing the accumulation of pesticides in the environment and organism (Campfens and Mackay, 1997).

The bioavailable fraction of any compound refers to the portion of the organism taken up by any organism from the total amount of the environment in which the compound is present. The nature of the composition, the nature of the sediment, and the organism's turbidity, as well as the bioavailability of contaminants in sediments, is generally in the range of 16-50% (Lamoureux and Brownawell, 1999).

Bioaccumulation refers to the taking of any chemical into the organism through all the ways (contact, gills, digestion, etc.). For example, predator animal tissues contain (pesticides, etc.) at higher levels contaminants than their prey (Bacci, 1994).

The structure and size of lipid tissues also affect the accumulation capacities of contaminants of different species and organisms. In the case of severe harshness or when the organism enters the stasis, the contaminants contained by using lipid deposits may release general circulation and show toxic effects (Landrum and Fisher, 1999).

Bellis is a fungicide commonly used to control plant pathogenic fungi in the south-eastern region of Turkey. In this study we aimed to determine the median concentration of Bellis to common carp (*Cyprinus carpio*). In addition we also estimate the level of Paraoxonase (PON1) enzyme, high-level density lipoprotein (HDL) and Malondialdehyde (MDA).

Li et al. (2003) reported that the amount of MDA in the liver of *Carassius auratus*, which has been exposed to 3,4 dichloroaniline for 15 days increased.

Durmaz et al (2005) investigated the effects of diazonin on antioxidant enzyme activities of various tissues of *O.niloticus* in their study. They found that diazonin caused an increment in MDA levels.

Oruç and Usta (2007) investigated the effects of diazonon on antioxidant enzyme activities in different tissiues of *C. carpio*. As a result of the study they reporte that diazonon caused and increment in MDA levels.

Dinçel et al. (2009) reported that there brain MDA levels increased after 1 week; on the other hand there was no significant difference in the amount of MDA in between control and cyfluthrin groups in the liver tissue of *C. carpio* exposed to sublethal dose of cyfluthrin.

Işık and Celik (2008) reported that MDA levels increased in *O. myciss*, exposed to methyl parathion and diazinon at 24, 48 and 72 hours.

Unver et al. (2014) have applied heavy metal (copper, zinc, cadmium) to zebrafish *(Danio rerio)* at different subletal doses. Lipid peroxidation in gill tissues after application Malondialdehyde (MDA), antioxidants such as glutathione (GSH), catalase (CAT) and total protein levels have been examined. As a result, even the lowest subletal doses of copper, zinc and cadmium have been shown to activate antioxidant defense mechanisms in the gill cells. Similar with the studies that used pesticides mentioned above it has been determined that the fungicide (Bellis) used in our study increases MDA which is an oxidative stress indicator and decreases HDL and PON which show antioxidant capacity. Parallel with the above studies, our findings showed that the antioxidant system decreased and the oxidant capacity increased.

Biochemical changes constituted in blood taken after 10 days from control group fishes not included ZnSO4 with (*Capoeta capoeta* [Guldenstaedt, 1773]) fishes added 5 and 10 mg/L ZnSO4 to life environments obtained that in plasma MDA levels ($P<0.001$) a statistically meaningful increase occurred in group II and group III when comparing to control group (Yilmaz et al., 2012)

Another study examines the levels of paraoxonase activity (PON1) and high-density lipoprotein (HDL) levels in the different doses of Capoeta capoeta administered zinc sulfate (ZnSO4). There was a decrease in PON1 activity and a decrease in HDL levels in the control group. (Deveci et al., 2015).

In our study, PON1 levels in control group was found significantly higher than Bellis 1 and Bellis 2 group ($p<0.001$). We think that Bellis, an organophosphate compound, may be responsible for the reduction of PON1 synthesis resulting from damage to the liver.

There are many studies on whether PON1 activity is related to the HDL cholesterol and apolipoprotein AI concentrations. PON1 activity is significantly reduced in Tangier disease and Fish eye disease with HDL deficiency, while there is no significant decrease in other HDL deficiencies (James et al., 1998).

PON1 is an enzyme associated with apo A1 and apo J (clustrein) proteins of HDL characterized by antioxidant properties (Hasselwander et al., 1998) and is able to metabolize peroxidized lipids and prevent the accumulation of lipid peroxides in both HDL and LDL (Mackness et al., 1991). Studies have suggested that HDL protects LDL against lipid peroxidation and is more effective than vitamin A and vitamin E (Mackness et al., 1991; Rousselot et al., 1999).

HDL is an active lipolipoprotein by function. In addition to its active contribution to cholesterol transport and metabolism, it will have an important defense role against diseases caused by microorganisms in carp, trout and possibly other teleost fish. In addition, HDL will retain the important defense function that preserves unchanging high concentrations in plasma even in diseased fish (Villarroel et al., 2007; Karataş and Kocaman, 2014).

Toxicity of organophosphate insecticides in vivo is carried out by enzymes such as Paraoxonase enzyme (PON1) acetylcholinesterase and carboxylesterase. Paraoxonase (PON1) is a serum esterase with the ability to hydrolyze paraoxon, an active metabolite of parathion, which is an organophosphorus insecticide synthesized in the liver (Gülcü and Gürsu, 2003). It has been reported that PON1, which is linked to HDL and has a strong effect on hydrolyzing lipid peroxides, protects HDL from oxidation (Aviram et al., 1998; Ekmekçi et al., 2004; Bayrak et al. 2005). It has been shown that Paraoxonase neutralize the atherogenic effects of lipid peroxides and to protect cell membranes. (Aviram et al., 2000; Ekmekçi et al., 2004).
In this study we found that PON1 activity decreased significantly ($p <0.001$) in the Bellis I and Bellis II groups (0.025 and 0.05 mg / L) compared to the control group.

In the Bellis I (0.025) group, PON 1 activity was higher, while it was observed to be even lower in the Bellis II group. Similarly, HDL levels were significantly reduced ($p < 0.001$) in the Bellis I and Bellis II groups (0.025 and 0.05 mg / L) compared to the control group. In the Bellis I (0.025) group, the HDL level was higher, while it was observed to be even lower in the Bellis II group.

Results indicate that:

- BELLIS can be change some biochemical parameters (PON1 , HDL and MDA) in the fish plasma samples.
- Bellis can be decrease level of antioxidant molecules and can be increase level of (MDA) oxidant molecule.
- Bellis can be increased PON1 and HDL levels in both Bellis groups compared to the control group.

The experiment that have done in agricultural activity declined, and the involved aquatic ecosystem Bellis fault due to reactive oxygen which is consisting of oxygen species and could improve oxidative stress and occur. For this reason it is believed to have a toxic effect.

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