The Effects of Endosulfan on Activity and Kinetic Properties of Lactic Dehydrogenase Enzyme: A Biochemical and Histopathological Study¶

ENDOSULFANIN LAKTİK DEHİDROGENAZ ENZİM AKTİVİTESİNE VE ENZİM KİNETİK ÖZELLİKLERİNE ETKİSİ

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Summary

Endosulfan insecticide is a polychlorinated compound that is common used in agriculture fields. In recent years in Turkey, especially in Çukurova Region, it has been suggested that there would be a relation between the increased incidence of breast cancer and widely use of endosulfan. For this purpose, effect of endosulfan on activity and kinetic properties of lactic dehydrogenase enzyme in erythrocyte, liver, and breast tissues of mature Mus musculus were investigated. Sixty mature (30 control, 30 experimental) M. musculus, weighing between 23 and 40 g, were obtained from the Medical Experimental Surgery Research Center of Çukurova University. The effects of oral administration of endosulfan (0.24 mg per 100 g body weight) daily for 90 days (short term) and for 180 days (long term) were investigated. As a result, no effect on body and breast weights whereas an increase on liver weight and hepato/somatic index were observed in both short and long terms. Activity of lactic dehydrogenase enzyme in erythrocyte, liver and breast tissues showed significant increase. Although the kinetic properties of lactic dehydrogenase enzyme of erythrocyte and liver tissues were effected by endosulfan; breast tissue kinetic properties showed no significant changes.

Key Words: Endosulfan, Mus musculus, Lactic dehydrogenase, Enzyme kinetic properties

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Özet

Poliklorlu bir bileşik olan endosulfan, tarım alanlarında yaygın olarak kullanılmaktadır. Son yıllarda Türkiye'de ve özellikle Çukurova bölgesinde meme kanseri riskinin artışı ile bölgemizde çok yaygın olarak kullanılan endosulfan arasında bir bağlantı olabileceği düşünüldüğünden, anaç Mus musculus'un eritrosit, karaciğer ve meme dokularında endosulfanın laktik dehidrogenaz aktivitesine ve enzim kinetik özelliklerine etkisi araştırılmıştır. Araştırmamızda Çukurova Üniversitesi Tıbbi Deneysel Cerrahi Araştırma Merkezi'nden alınan 23-40 g ağırlığında 60 adet (30 kontrol, 30 deneysel) anaç M. musculus kullanılmıştır. Deneysel gruba endosulfan (0.24 mg/100 g vücut ağırlığı/gün) 90 gün (kısa dönem) ve 180 gün (uzun dönem) oral yolla uygulanmıştır. Endosulfanın hem kısa hem de uzun dönemde vücut ve meme ağırlıklarını etkilemediği, karaciğer ağırlığını ve hepato/somatik indeksi arttırdığı ve eritrosit, karaciğer ve meme dokularında laktik dehidrogenaz aktivitesini arttırdığı saptanmıştır. Endosulfanın, eritrosit ve karaciğer dokusu laktik dehidrogenaz enzim kinetik özelliklerini belirgin şekilde etkilerken meme dokusunu etkilemediği gözlenmiştir.

Anahtar Kelimeler: Endosulfan, Mus musculus, Laktik dehidrogenaz, Enzim kinetik özellikleri

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Endosulfan (6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6, 9-methano-2, 4, 3-benzodiexathiepin-3-oxide), a broad spectrum insecticide of the cylodiene group, is a potential enviromental pollutant (1). It is metabolized in the liver as a lipophilic xenobiotic to hepatotoxic intermediates by monooxygenases (2,3). The free radicals generated during the metabolism of endosulfan are quenched by the antioxidant system present in the living cell as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GSH-R), glutathione peroxidase (GSH-P), reduced glutathione (GSH)

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and eventually the final electron acceptor NADPH generated by G6PDH (4,5).

Lactic dehydrogenase (LDH) is an enzyme in the glycolytic pathway and is released as the result of cell damage. LDH activity is present in all cells of the body and is invariably found only in the cytoplasm of the cell. Elevations of LDH activity are observed in various diseases as well as in cancer (6).

Endosulfan causes increase in G6PDH activity, blood glucose levels, phospholipid contents of the microsomal and surfactant system, and profoundly induce the activity of the alcohol dehydrogenase and cytosolic glutathione-S-transferases. It also decreases significantly Na-K and Mg ATPases, plasma calcium level and alkaline phosphatase in the intestinal epithelium (1).

In the recent years in Turkey, especially in Çukurova Region, it has been suggested that there would be a relation between the increased incidence of breast cancer and widely use of endosulfan. For this purpose, the effect of endosulfan on the activity and the kinetic properties of LDH enzyme in erythrocyte (RBC), liver, and breast tissues of mature mice (Mus musculus) were investigated.

Material and Methods

Chemicals: Reduced nicotinamide adenine dinucleotide (βNADH), reduced nicotinamide hypoxantine dinucleotide (β-dNADH), bovine serum albumin (BSA), nitroblue tetrazolium (NBT) and phenazine methosulfate (PMS), nicotinamide adenine dinucleotide (βNAD) were obtained from the Sigma Chemical Co. DE-52 was from Whatman (Maidstone, England). All the other chemicals were analytical grade products of Merck (Darmstadt, Germany).

Animals: Non-inbred M. musculus albino, sixty mature, weighing between 23 and 40 g were obtained from the Medical Sciences Experimental Research Center of the University of Çukurova. They were fed a standard laboratory diet and tap water. Illumination was 12 hour light/dark cycle and room temperature was 22-24ºC. The control group consisted of thirty apparently normal M. musculus and thirty were exposed to endosulfan. The experimental group was divided into two groups (Group I and II) and were exposed to endosulfan as oral administration (0.24 mg per 100 g body weight) daily for a 90 days and 180 days period, respectively. Blood was extracted from the tail vein and, after the experiment, from the heart. As soon as the M. musculus was sacrificed, the liver and breast tissues were quickly removed, weighed and homogenized with three volumes of ice-cold %1.15 M KCI. The activity of LDH enzyme was measured in 14.000 x rpm supernatant.

Enzyme Activity: Activity was determined at 37ºC in the hemolysate and homogenate according to procedure of Beutler (7). Protein concentrations were measured using BSA as the standard (8) and hemoglobin was determined by the cyanomethemoglobin method (7). The specific activity of the enzyme was calculated for hemolysate as U/g Hb and for homogenate as U/mg protein.

Biochemical Parameters: Kinetic studies were made in accordance with the procedure of the WHO scientific group (9). LDH enzyme from M. musculus RBC, liver and breast tissues was partially purified by ion exchange with DE-52 as ligand. The steady-state kinetic parameters were obtained spectrophotometrically using a Shimadzu U.V 260 instrument. Michaelis constants (Km) for Pyruvate and NADH were determined in partially purified enzyme at 37ºC. Pyruvate concentrations ranged from 7.5 to 300 µmol and NADH concentrations 1 to 40 µmol in the Km studies. The utilization rates of dNADH was measured. The heat stability of LDH enzyme was determined after 10 and 20 minutes incubation at 46ºC (10). Polyacrylamide gel electrophoresis (PAGE) was performed using 0.08 M Tris-HCl buffer, pH 8.0, at room temperature. Before electrophoresis, samples were dialyzed 2 hours at + 4ºC against running buffer. The active band was stained by the staining solution containing 0.3 mM PMS, 2.8 mM NBT, 2mM NAD in 10 ml. The gel was destined in 10% acetic acid at room temperature. The protein concentration of the liver and breast tissues was determined in a Shimadzu UV 120-02 spectrophotometer following the procedure of Lowry using BSA as a standard.

Histopathology: The liver and breast tissues were fixed in 10% formaldehyde and processed routinely. They were embedded in paraffin. Five µm sections were obtained, stained with Harris hematoxylene-eosin and examined under light microscope (11).

Statistics: The SPSSX programme was used for Wilcoxon-Mann-Whitney rank sum test (U test). Results were expressed as the means ± standard deviation (SD).

Results

Whereas endosulfan had no significant effect on the total body and breast weights ($p>0.05$) of the mice, it did increase the weight of the livers and the hepato/somatic index (HSI: liver weights/body weights) significantly (Table 1). The effects of endosulfan on LDH activities in the RBC, liver and breast tissues are shown in Table 2. Figure 1 shows the results of the thermal inactivation studies in RBC and liver of the control and the experimental groups. Thermal inactivation properties of the control and the experimental groups were also significantly different. The relative utilization of β-dNADH, significantly different as shown in Table 3. PAGE of both the control and the experimental groups gave a single homogenous band and no differences in electrophoretic mobility were noted. The kinetic properties of breast LDH enzyme studies were not different in the control and the experiment groups (Table 3).

Histopathological examinations of liver tissues of group I animals showed chronic toxic hepatitis in liver. There was portal mononuclear inflammatory infiltration and some eosinophyl leucocytes and lobulary inflammation (liver cell necrosis). There wasn't neoplastic and dysplastic changes in liver (Figure 2). Histopathological examinations of liver tissues of group II animals showed some regenerative findings with mild hepatitis. Hepatocytes had more than one nucleus, nuclear hyperchromasy and minimal microvesiculary fatty degeneration. In addition, we saw crude glycogen granules in hepatocytes (Figure 3). Distribution of lesions in liver tissues of experimental animals are shown in Table 4.

Microscopic examinations of breast tissues of group I and II animals showed lymphocytic infiltration in stroma of breast tissue. There was no neoplastic and dysplastic changes in breast (Figure 4).

Tablo 1. Liver weights and HSI in control and experimental groups

	n	Liver weights (g)	HSI
Control	30	0.742±0.208	0.021±0.007
I	15	*1.144±0.296	*0.034±0.010
II	15	*1.179±0.270	*0.035±0.010

**Liver weights and HSI increased significantly in both short and long terms of exposure to endosulfan compared to control group ($p<0.05$) (Mann-Whitney-U test).*

Table 2. LDH enzyme activities in RBC, liver and breast tissues

	n	LDH activities
RBC (U/g Hb)		
Control	30	281.0±52.5
Group I	15	*497.6±115.7
Group II	15	*868.0±101.4
Liver tissue (U/mg protein)		
Control	30	2.3±0.7
Group I	15	*9.4±1.6
Group II	15	*4.6±1.0
Breast tissue (U/mg protein)		
Control	30	4.5±1.1
Group I	15	*6.9±1.8
Group II	15	*6.0±1.3

** LDH activities increased in experimental group in both short and long terms compared to control ($p<0.05$) (Mann-Whitney-U test).*

Discussion

Endosulfan is metabolized in the liver through cytochrome P450 system to hepatotoxic intermediates. The highly reactive free radicals generated during the course of the reaction cause damage to the organism (1,3). In this study, we have observed the toxic effects of short and long terms exposure to endosulfan pesticide on the RBC, liver and breast tissues. As shown in Figure 2 and 3, the livers of group I and II suffered histopathological damage. The increase in weight of livers and HSI was significant and greatest in the long term pesticide exposure with no further change on short term. According to Gill et al. (12), HSI of fresh water fish, Barbus conchonius was moderately increased after 2, 3 and 4 weeks of exposure to 6.72 ppb of organochlorine insecticide endosulfan. Chlorinated hydrocarbon insecticides (DDT, endosulfan, etc.,) cause liver damage that ranges from increased liver weights and fat content to cell necrosis (13). However, some enzyme activity changes are relat-

Table 3. The kinetic properties of LDH enzyme in experimental and control groups

		Km (μmol)		Utilization (%)	Heat stability (%) activity remaining after 20 min. incubation
		Pyruvate	NADH	DNADH	
RBC	Control	73.70±14.93	7.20±1.98	65.70±14.86	87.70±10.33
	Group I	*40.10±8.29	*20.40±7.61	*218.10±63.93	*54.30±11.57
	Group II	*27.60±10.00	*27.60±10.02	*204.90±63.01	*34.80±8.40
Liver	Control	52.80±12.25	14.30±4.19	78.40±21.79	89.20±12.07
	Group I	*36.00±10.24	*27.90±9.17	*244.60±103.11	*54.50±10.64
	Group II	*26.70±5.03	*16.90±4.06	*182.30±62.86	*26.70±5.03
Breast	Control	54.40±20.09	10.10±3.92	60.70±10.99	84.00±11.14
	Group I	43.10±13.74	9.00±3.80	62.80±10.80	72.00±22.40
	Group II	50.50±18.99	10.80±3.92	60.00±10.98	81.80±11.46

10 animals for each the groups used for the kinetic properties of LDH enzyme in the various tissues.
**The kinetic properties of LDH enzyme were different in RBC and liver of the experimental and control groups (P<0.05) (Mann-Whitney-U test).*

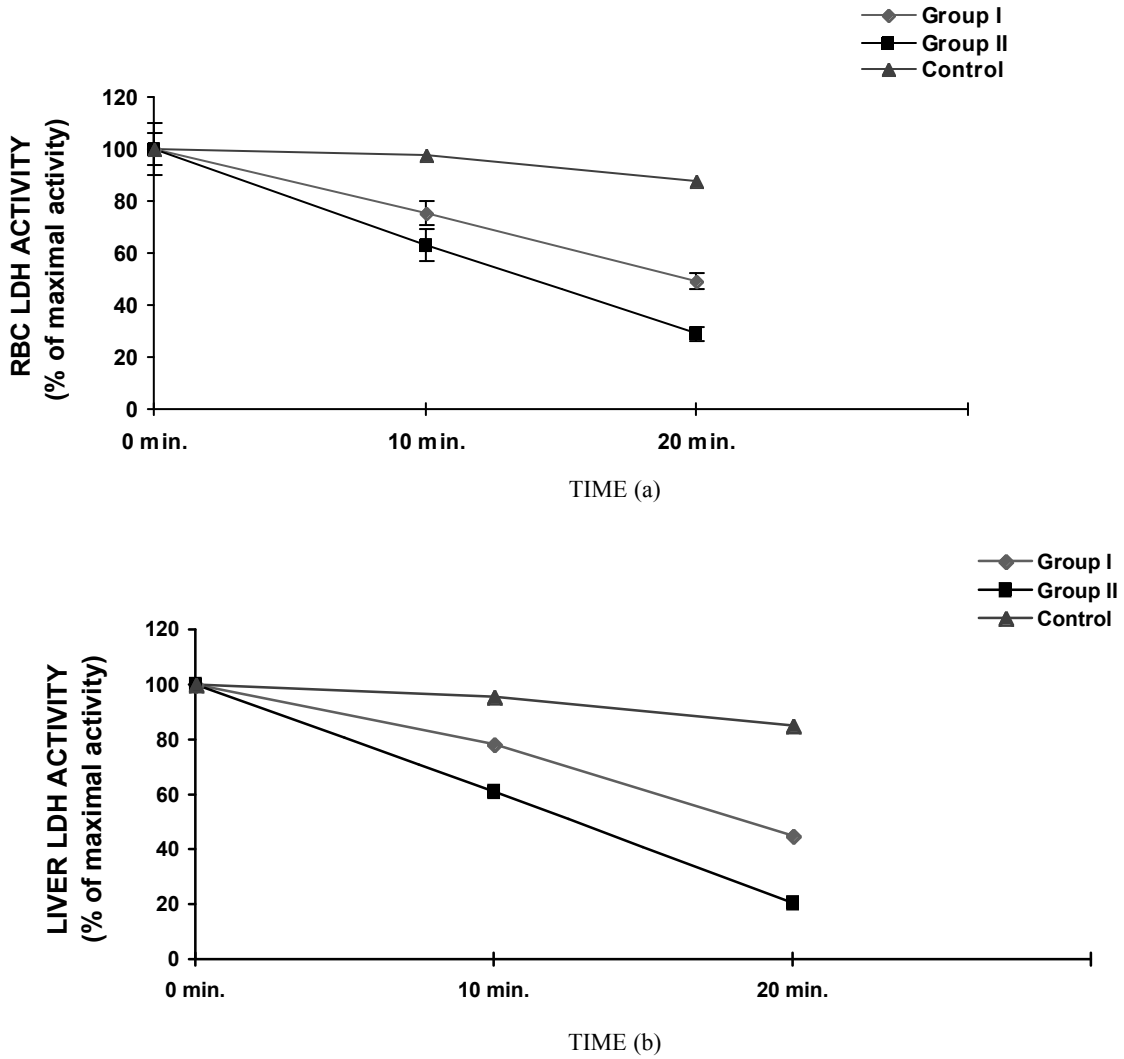

Figure 1. Heat stability of RBC and liver LDH enzyme in experimental groups decreased significantly compared to control (p<0.05) (Mann- Whitney-U).

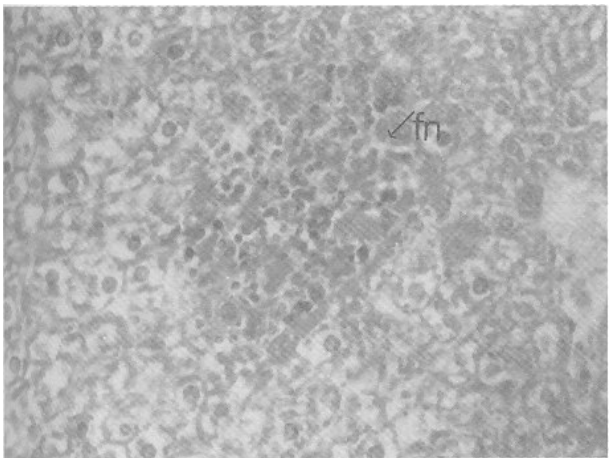

Figure 2. Liver tissues taken from group I of Mus musculus albino mouse (Hematoxylene-Eosin x200) fn: focal necrose.

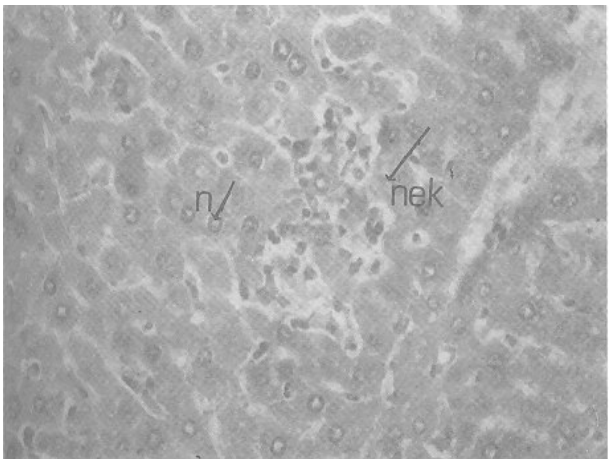

Figure 3. Liver tissues taken from group II of Mus musculus albino mouse (Hematoxylene-Eosin x200) nek: necrosis of single cell, n: vesicular appearance in the nucleous of hepatocyte.

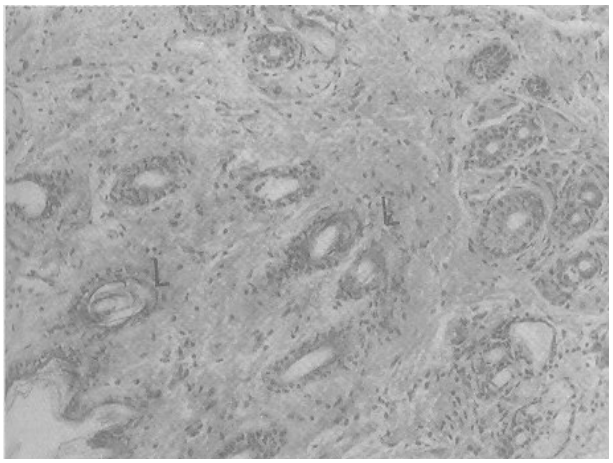

Figure 4. Breast tissues taken from group I and II of Mus musculus albino mouse (Hematoxylene-Eosin x200) L: lymphocyte infiltration.

ed to hepatic alterations induced by pesticides, including induction of serum amino transferases, lactic dehydrogenase and alkaline phosphatases. Damage to liver cells leads to changes in the organelles (14). These morphological changes disturb various biochemical reactions which then can be measured in the serum or RBC. In this study, the RBC LDH enzyme exhibited a significant increase in short term with further increase in long term. Asztalos et al. reported that the RBC LDH activity of carp sera fish, Cyprinius carpio L. was significantly increased after 5 ppm treatment copper sulphate, paraquat and methidation pesticides over a period of 1-2 weeks (15). This pesticides cause necrosis in the heart and skeletal muscle tissue. As with the G6PDH, the LDH enzyme in RBC might be showing a conformational change whereas in the liver there might be a change as a result of genetic control. But in the liver, the increase in liver LDH enzyme activity was greatest in short term whereas in the breast tissue the increase was in short term with no further change in long term. In our experiments we have partially purified and characterized the enzymes obtained from the RBC, liver and breast tissues of M. musculus exposed do the short and long term of endosulfan. The Michaelis constant (Km) of the substrate pyruvate, and, cofactor NADH were different in the RBC and liver of control and the experimental groups but they were not different in breast tissues. The Km of the RBC and liver for pyruvate was lower than control group for group I but it was even lower for group II; whereas the Km for NADH was higher than control group for group I but it was increased for group II. Also, the heat stability of the RBC and liver enzymes in experimental group was lower than control group. Electrophoretic mobilities of in control and experimental groups were found to be similar. However, the RBC and liver enzymes in the exprimental groups showed an increased utilization of dNADH. Thus the results obtained from group I and II had significantly different biochemical properties from the liver and RBC enzymes of control group. These data shown that endosulfan had significant effect on kinetic properties of RBC and liver LDH enzymes whereas it did not effect significantly in breast tissues.

Our results suggested that exposure of endosulfan to mice caused liver tissue necrosis by in-

Table 4. Hepatic injury produced by endosulfan in experimental animals

	Necrosis	Fatty degeneration	Inflammation Z_1	Z_2	Z_3	Granuloma	Cholestasis	Finding of Regeneration	Dysplastic changes	Neoplastic changes
Group 1	6	11	15	6	-	-	4	8	-	-
Group 2	8	10	5	9	-	-	6	15	-	-

Z_1: Zone 1 Z_2: Zone 2 Z_3: Zone 3

creased levels of LDH. We thought that LDH enzyme may be an important biochemical marker for pesticide toxicity to mammalian and also applicable to human tissues.

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REFERENCES

1. Maqvi SM, Vaishnai C. Bioccumulative potential and toxicity of endosulfan insecticide to non-target animals. Comp Biochem Physiol 1993; 105: 347-61.
2. Hunter DJ, Kelsey KT. Pesticide residues and breast cancer: the harvest of a silent spring. J Natl Cancer Inst 1993; 85: 598-9.
3. Kitchin KT. An enzymatic approach to biotransformation. Meth Find Exptl Clin Pharmacol 1984; 6: 303-10.
4. Liang HCL, Shertzer HG, Nebert DW. "Oxidative stress" responce in liver of an treated newborn mouse having a 1.2-centimorgan deletion on chrosome 7. Biochem Biophys Res Commun 1992; 182: 1160-64.
5. Belge E, Oruç E, Yüregir GT, Üner N, Doran F, Varinli S. Mus musculus and Oreochromis niloticus'ta karaciğer dokusunda antioksidan sistemlerin karşılaştırılması. Erciyes Tıp Derg 1997; 4: 239-43.
6. Tietz W. Fundamentals of Clinical Chemistry. Philadelphia: WB Saunders Comp. 1999: 787-90.
7. Beutler E. Red Cell Metabolism: A Manual of Biochemical Methods. In: Grune and Stratten, eds. New York: Book Comp. 1975: 265-75.
8. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol. J Biol Chem 1951; 193: 265-75.
9. Report of a WHO Scientific Group. WHO Tech Rep Ser 1967; 366: 5-53.
10. Davis BJ. Disc electrophoresis II: Method and application to human serum proteins. Ann NY Acad Sci 1964; 121:404-9.
11. Bancroft JD, Stevens A. Theory and Practice of Histological Techniques. Edinburgh: Churchill Livingstone, 1977: 3-51.
12. Gill TS, Pande J, Tewart H. Effects of endosulfan on the blood and organ chemistry of freshwater fish. Ecotoxicol Enviromental Saf 1991; 21:80-91.
13. Matsumura F. Toxicology of Insecticides. New York: Plenum Press, 1985: 598-627.
14. Götz W. Diagnosis of Hepatic Disease. Germany: GITV Verlag Ernst Giebeler, 1981: 64-107.
15. Cevi AP, Rao DMR, Tılak KS, Murty AS. Relative toxicity of the technical grade materials, isomers and formulations of endosulfan of the fish, Channa punctatus. Bull Environ Contam Toxicol 1981; 27: 239-43.
16. Asztalos B, Nemcsok J. Effects of pesticides on the LDH activity and isoenzyme pattern of carp (Cyprinus carpio L.) sera. Comp Biochem Physiol C 1985; 82: 217-9.