

Protective and Antigenotoxic Effects of Silymarin and Curcumin in Experimental Cyclophosphamide Intoxication in Rats ^[1]

Hamdi AVCI ¹  Selim SEKKİN ² Murat BOYACIOĞLU ² Hasan AKŞİT ³
Recai TUNCA ¹ E. Tuğrul EPİKMEN ¹ S. Serap BİRİNCİOĞLU ¹

^[1] This study was presented at the VII. National Congress of Pathology, Sept 8-10, 2014, Kars/Turkey, and supported by the Scientific and Technological Research Council of Turkey (TUBİTAK); Project number: 111O521

¹ Department of Pathology, Faculty of Veterinary Medicine, University of Adnan Menderes, TR-09016 Aydın - TURKEY

² Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Adnan Menderes, TR-09016 Aydın - TURKEY

³ Department of Biochemistry, Faculty of Veterinary Medicine, University of Balıkesir, TR-10100 Balıkesir - TURKEY

Article Code: KVFD-2016-15145 Received: 29.01.2016 Accepted: 05.04.2016 Published Online: 05.04.2016

Abstract

The aim of this study was to investigate the protective effects of silymarin (SLY) and curcumin (CUR), which have strong antioxidant properties, against the toxic effects of high dose of cyclophosphamide (CP) on the liver and kidneys of rats. For this purpose, a total of 36 adult Wistar albino female rats at the weight of, 250±10 g were used. Biochemical parameters were found to be significantly higher in the CP-only group than in the other groups. In the groups in which SLY and CUR were administered concurrently with CP, biochemical parameters were found to be significantly lower than in the CP-only group. In the SLY+CP and CUR+CP groups, biochemical parameters were correlated with the severity of histopathological findings. In genotoxic examination, the comet parameter levels in the SLY+CP and CUR+CP-treated groups were lower than those in the CP-treated group. The present study established that both SLY and CUR are effective against the toxic effects of high doses of CP.

Keywords: Cyclophosphamide, Silymarin, Curcumin, Biochemistry, Pathology, Comet assay

Ratlarda Deneysel Siklofosfamid Toksikasyonunda Silimarin ve Curcuminin Koruyucu ve Antigenotoksik Etkileri

Özet

Bu çalışmada, yüksek dozda siklofosfamid (CP) verilen ratların karaciğer ve böbrekler üzerindeki toksik etkilerine karşı kuvvetli antioksidan özellikleri bulunan silimarin (SLY) ve curcuminin (CUR) koruyucu etkileri araştırıldı. Bu amaçla çalışmada toplam 36 adet, ağırlıkları 250±10 gram olan erişkin Wistar albino ırkı dişi ratlar kullanıldı. Yalnızca CP verilen grupta biyokimyasal parametreler kontrol grubuna göre istatistiksel olarak anlamlı bir şekilde yüksek bulundu. CP ile birlikte eş zamanlı olarak SLY ve CUR verilen gruplarda biyokimyasal parametreler, yalnızca CP verilen gruba göre istatistiksel olarak anlamlı bir şekilde düşük bulundu. Genotoksik incelemede comet parametreleri CP ile birlikte eş zamanlı SLY ve CUR verilen gruplarda ise yalnızca CP verilen gruba göre istatistiksel olarak anlamlı bir şekilde düşük bulundu. Çalışmada CP'nin toksik etkilerine karşı, kuvvetli antioksidan özelliklere sahip SLY ve CUR'un koruyucu alternatif birer kimyasal madde olarak kullanılabileceği görüldü.

Anahtar sözcükler: Siklofosfamid, Silimarin, Curcumin, Biyokimya, Patoloji, Comet assay

INTRODUCTION

Cyclophosphamide (CP) (*N,N*-bis(2-chloroethyl tetrahydro-2H-1,3,2-oxphosphorin-2-amine, 2-oxide monohydrate), also known Cytoxan or Endoxan, is a pharmaceutical product used as an antineoplastic agent in the treatment of a wide range of cancers, including

Hodgkin's disease, many types of leukaemia, multiple myeloma and neuroblastomas ^[1,2]. Despite its therapeutic importance, a wide range of adverse effects, including nephrotoxicity ^[2], hepatotoxicity ^[3], cardiotoxicity ^[4] and male reproductive toxicity ^[5], have been reported for the drug.

Since the 1980s, medicinal plants and their active ingredients have received increasing attention ^[6]. Medicinal



İletişim (Correspondence)



+90 256 2470700/234



havci@adu.edu.tr

plants serve as therapeutic alternatives, safer options or, in some cases, the only effective treatment, and an increasing number of these plants and their extracts have been shown to produce beneficial therapeutic effects, including antioxidant, anti-inflammatory, anticancer, antimicrobial and immunomodulatory effects [7-9]. Among the promising medicinal plants, silymarin (SLY) [10-12] and curcumin (CUR) [7,8,13] have been reported to have multiple pharmacological activities, including antioxidant, hepatoprotectant and anti-inflammatory, antibacterial, and antineoplastic effects. SLY is the polyphenolic fraction from the seeds of milk thistle (*Silybum marianum*) and is composed of various flavonoids, including silybin (major component), silydianin, and silychristine [14]. It has been reported that silymarin acts as an antioxidant, reducing free-radical-mediated injury in tissues and inhibiting lipid peroxidation [14,15]. CUR is a hydrophobic polyphenol derived from the rhizome of herb *Curcuma longa* belonging to family zingiberaceae and has been used for centuries in indigenous medicine [16]. A large body of evidence suggests that CUR has a diverse range of molecular targets, including transcription factors, growth factors, and their receptors, cytokines, enzymes, and genes regulating cell proliferation and apoptosis [13,16].

The aim of the present study was to evaluate and compare the protective roles of SLY and CUR in CP-induced hepatotoxicity, nephrotoxicity and genotoxicity in rats.

MATERIAL and METHODS

Chemicals, Animals and Experimental Design

CP (Endoxan) was purchased from Eczacıbaşı-Baxter (Istanbul, Turkey). SLY (code; S0292) and CUR (code; C1386) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Thirty-six healthy adult female Wistar albino rats (3 months old, 250±10 g body weight) were used. The rats were obtained from Adnan Menderes University Experimental Research Centre (Aydın, Turkey) and were housed under standard laboratory conditions (24±3°C, 40-60% humidity, 12-h light-dark cycle). A commercial pellet diet and fresh drinking water were available *ad libitum*. All animals received humane care according to criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health, and the experimental protocol was approved by the University of Adnan Menderes Institutional Animal Ethics Committee (No: B.30.2.ADU.0.00.00.00/050.04/2011/043).

Rats were randomly divided into six experimental groups containing six rats each, as follows: Group I (control group; nothing was administered), Group II (CP group; 30 mg/kg/day CP administered intraperitoneally to each animal for seven days), Group III (SLY group; 100 mg/kg/day SLY by gavage for 14 days), Group IV (CUR group; 100 mg/kg/

day CUR by gavage for 14 days), Group V (SLY + CP group; 100 mg/kg/day SLY by gavage for 14 days plus 30 mg/kg/day CP intraperitoneally starting from the eighth day) and Group VI (CUR + CP group; 100 mg/kg/day CUR by gavage for 14 days plus 30 mg/kg/day CP intraperitoneally starting from the eighth day). All administrations were applied by gavage as an emulsion in 0.5 mL corn oil.

Biochemistry

The rats were killed under slight ether anaesthesia at the end of 14 days. Blood samples were collected and then transferred directly into plain vacutainer tubes, centrifugated at 1.200 g for 10 min at 4°C and stored at -20°C until analysis. Serum ALT (alanine aminotransferase, Archem, A2221, Turkey), AST (aspartate aminotransferase, Archem, A2212, Turkey) and creatinine (Archem, A2162, Turkey) were assayed using a Biochemistry Auto Analyzer (Sinnowa D280, China).

For the determination of malondialdehit (MDA) levels and superoxide dismutase (SOD) activity in the liver and kidneys, the dissected tissues were immediately rinsed in ice-cold phosphate-buffered saline. Tissues were homogenised (2000 rpm/min for 1 min, 1/10 w/v) using a Teflon-glass stirrer (IKA Overhead Stirrer; IKA-Werke GmbH & Co. KG, Staufen, Germany) in a 10% 150 mM phosphate buffer (pH 7.4) in an ice bath. The homogenate was centrifuged (Hettich Zentrifugen, Mikro 200 R, Tuttlingen, Germany) at 6.000 g for 10 min at 4°C. The supernatants were frozen at -80°C (Glacier Ultralow Temperature Freezer, Japan) until analysed and then used for determination of MDA levels and SOD activity. The lipid peroxidation levels were determined according to the concentration of thiobarbituric acid reactive substances, and the amount of MDA produced was used as an index of lipid peroxidation. Absorbance was measured with a spectrophotometer at 532 nm. The MDA concentration was calculated by the absorbance complex (absorbance coefficient $\epsilon = 1.56 \times 10^5$ /M/cm) and expressed as nmol/mg of tissue protein [17]. SOD activity was determined according to the method of Sun *et al.* [18], and the absorbance was measured with a spectrophotometer at 560 nm. This method is based on the inhibition of nitro blue tetrazolium reduction using the xanthine-xanthine oxidase system as a superoxide generator. SOD activity was then measured by the degree of inhibition of this reaction. The protein levels in the tissues were determined by the method described by Lowry *et al.* [19].

For the measurement of DNA fragmentation levels, the liver and kidneys samples were removed and protected against light, and then stored at -20°C until analyses. The extent of apoptosis was evaluated by the measurement of DNA fragmentation. This was assessed by quantification of cytosolic oligonucleosome-bound DNA by using the Cell Death Detection ELISA Plus kit (Roche, Mannheim, Germany). The liver and kidneys of rats were treated with a homogeniser (Stuart SHM1, UK). The 0.2 g homogenate

was made with the lysis buffer and then centrifuged at 20.000 g for 10 min at 4°C. The supernatant fraction was used as the antigen source for the immunoassay. This assay is based on the quantitative sandwich ELISA principle using mouse monoclonal antibodies directed against histones (coating antibody) and DNA (peroxidase-labelled antibody), respectively. The amount of peroxidase retained in the immunocomplex is determined photometrically with ABTS (2,29-azino-di-(3-thylbenzthiazoline sulfonate) as a substrate (Thermo Multiskan FC Microplate Photometer, USA). This allows the specific determination of mono- and oligo-nucleosomes in the cytoplasmic fraction of cell lysates.

Histopathology

For histopathological examinations, samples of the liver and kidneys were taken. The liver was cut into thinner sections and each kidney was cut into two pieces along its transverse axis. Parts of the liver and kidneys were fixed in 10% neutral buffered formalin. After fixation, sections were embedded in paraffin wax and sectioned at 4-6 µm and stained with haematoxylin-eosin. The histopathological results were semiquantitatively assessed under a light microscopy with ocular grids and x 10, x 20 and 40 objectives. In total, 10 high-power fields were randomly chosen in each section. Changes in the experimental histopathological findings were graded as follows: 0, no changes, 1, mild changes, 2, moderate and 3, severe changes [20].

TUNEL Assay

Apoptotic cells were detected using the DeadEnd Colorimetric TUNEL system following the manufacturer's protocol (GenScript, USA). In brief, tissue sections after deparaffinisation and rehydration were permeabilised with proteinase K for 30 min at ambient temperature. Thereafter, the sections were quenched of endogenous peroxidase activity using 0.3% hydrogen peroxide for 10 min. After thorough washing with 3 x phosphate buffered saline (PBS), sections were incubated with equilibration buffer for 10 min, and terminal deoxynucleotidyl transferase reaction mixture was then added to all the sections, except for the negative control, and incubated at 37°C for 1 h. The reaction was stopped by immersing the sections in 2x saline sodium citrate buffer (SSC) for 15 min. Sections were then treated with conjugated horseradish peroxidase streptavidin (1:500) for 30 min at room temperature, and after repeated washing, sections were incubated with 3,30-diaminobenzidine for 90 s for colour development. Sections were mounted after dehydration and observed under 400 x magnification for TUNEL-positive cells. The results of the TUNEL assay were assessed using a grading system based on the total number of positive cells. For quantification of the TUNEL assay, the tissue areas with the highest density of positive staining were chosen. All positive cells from a total of 10 high-power fields measured 0.025 mm² were counted. To calculate the total number of positively stained cells, an ocular grid of 100 (10x10)

squares was used at 20X microscope objective.

Lymphocyte Isolation and DNA Analysis by Comet assay

The comet assay used here was adapted from the method described previously [21-23]. For this purpose, fresh blood samples were mixed with the PBS solution for the determination of DNA fragmentation of blood lymphocytes. Lymphocytes were isolated with histopaque and suspended in a freezing medium. Isolated lymphocytes were slowly frozen in aliquots of 1 ml at - 80°C. Conventional end-frosted slides were pre-coated with 1% normal melting agarose. This suspension was mixed with pre-warmed low - melting - point agarose. The positive control slide cells were dipped in an H₂O₂ solution for 5 min at 4°C. Following lysis, slides were aligned in a horizontal gel electrophoresis tank (CSL - COM20, Cleaver Scientific, UK) that was connected to a recirculating cooler (FL300, Julabo, Germany) set at 4°C and filled with freshly made alkaline electrophoresis solution. Electrophoresis (CS - 300V, Cleaver Scientific, UK) was carried out at approximately 1 V/cm for 20 min. Measurements of the tail intensity and tail moment of comets were made using a computer-based image analysis system (Comet Assay IV, Perceptive Instruments, UK). The mean value of the % Tail DNA and Mean Tail Moment parameters was calculated and used to assess the DNA damage.

Statistical Analysis

All data were checked for normal distribution with the Shapiro-Wilk test and for homogeneity of variance with Levene's test. The data were compared among groups using the Kruskal-Wallis analysis of variance (ANOVA) or one-way ANOVA, according to whether data were normally distributed or not. Post hoc multiple comparisons were performed using the Mann-Whitney *U* test with Bonferroni corrected or Duncan's test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software. Differences were considered statistically significant if $P < 0.05$, $P < 0.01$ and $P < 0.001$. All data were expressed as mean and standard error [24].

RESULTS

Biochemistry

The levels of biochemical parameters including liver (ALT and AST) and kidney (creatinine) enzymes, the lipid peroxidation (MDA) and antioxidant (SOD) levels, and DNA fragmentation (ELISA) in the liver and kidneys of all groups are presented in *Table 1*. A significant increase in serum ALT, AST and creatinine levels were observed in the CP-treated group compared to the control and other treated groups. However, it was observed that AST and creatinine activity in the SLY+CP- and CUR+CP-treated groups were significantly lower than those in the CP group.

Table 1. Biochemical Parameters, TUNEL and Comet assay results in all groups**Tablo 1.** Tüm gruplara ait biyokimyasal parametreler, TUNEL ve Comet assay sonuçları

Biochemical Parameters, TUNEL and Comet Assay Results	Experimental Groups						P
	Control	CP	SLY	CUR	SLY+ CP	CUR+ CP	
Liver							
ALT (U/L)	20.33±5.16 ^b	30.16±2.60 ^a	16.00±2.08 ^b	15.50±1.33 ^b	22.00±1.39 ^b	21.66±0.95 ^b	**
AST (U/L)	61.16±6.75 ^c	147.33±5.67 ^a	62.66±3.41 ^c	57.66±6.37 ^c	125.66±0.95 ^b	117.00±3.95 ^b	***
MDA(nmol/mg protein)	47.54±2.32 ^{b,c,d}	110.13±2.49 ^a	45.16±2.18 ^{c,d}	41.66±3.22 ^d	53.91±1.54 ^b	52.09±3.14 ^{b,c}	***
SOD (U/mg protein)	4.02±0.22 ^{ab}	2.69±0.14 ^c	4.21±0.18 ^{ab}	4.40±0.12 ^a	3.72±0.15 ^b	3.97±0.20 ^{ab}	***
DNA fragmentation (U/mg protein)	0.81±0.04 ^b	1.49±0.06 ^a	0.78±0.07 ^b	0.74±0.13 ^b	1.06±0.09 ^b	1.03±0.20 ^b	**
TUNEL	8.00±0.00 ^c	20.00±0.00 ^a	5.33±0.33 ^d	5.00±0.00 ^d	15.00±0.00 ^b	10.00±0.00 ^b	***
Kidney							
Creatinine (mg/dl)	0.65±0.14 ^c	1.27±0.05 ^a	0.41±0.13 ^c	0.40±0.13 ^c	0.96±0.05 ^b	0.95±0.02 ^b	***
MDA (nmol/mg protein)	87.41±3.68 ^{c,d}	188.08±3.87 ^a	86.98±3.14 ^{c,d}	81.66±2.22 ^d	107.57±3.43 ^b	98.46±5.71 ^{b,c}	***
SOD (U/mg protein)	5.00±0.15 ^b	2.05±0.25 ^c	5.18±0.22 ^{ab}	5.97±0.40 ^a	4.51±0.35 ^b	4.71±0.19 ^b	***
DNA fragmentation (U/mg protein)	1.09±0.03 ^c	2.82±0.17 ^a	1.22±0.02 ^b	1.09±0.03 ^c	1.50±0.10 ^b	1.41±0.15 ^b	***
TUNEL	9.33±0.42 ^c	62.50±2.14 ^a	7.50±0.22 ^d	7.33±0.21 ^d	33.33±1.05 ^b	22.50±1.11 ^b	***
Comet Assay							
Olive tail moment	22.83±3.06 ^b	46.03±1.18 ^a	15.35±1.46 ^c	11.76±1.18 ^c	28.20±4.22 ^b	17.17±2.75 ^{b,c}	***
Tail DNA (%)	54.94±4.11 ^b	82.71±1.73 ^a	50.63±2.85 ^b	42.06±3.24 ^{b,c}	62.78±4.92 ^b	52.94±7.35 ^b	***

^{a, b, c, d} Different letters indicate statistically significant differences in the same row, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$; CP: Cyclophosphamide, SLY: Silymarin, CUR: Curcumin

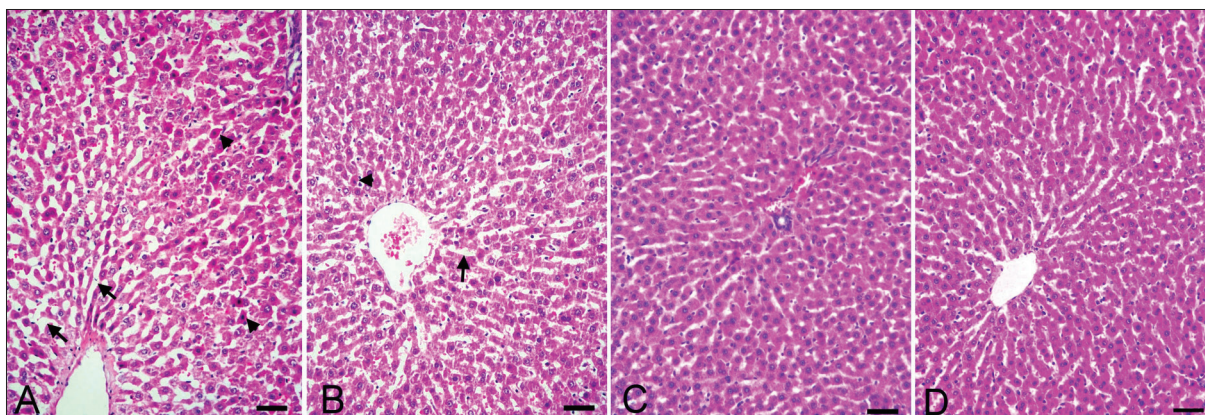


Fig 1. Liver. A. Severe dilatations in sinusoids (arrows) and eosinophilic staining in hepatocytes (arrowheads), CP group, HE, Bar: 50 μ m, B. Mild dilatations in sinusoids (arrow) and eosinophilic staining in hepatocytes (arrowhead), CUR + CP group, HE, Bar: 50 μ m, C. Normal histological appearance of liver, CUR group, HE, Bar: 50 μ m, D. Histological appearance of liver in control group, HE, Bar: 50 μ m

Şekil 1. Karaciğer. A. Sinuzoidlerde şiddetli dilatasyon (oklar) ve hepatositlerde eozinofilik boyanma (okbaşları), CP grup, HE, Bar: 50 μ m, B. Sinuzoidlerde hafif dilatasyon (ok) ve hepatositlerde hafif eozinofilik boyanma (okbaşı), CUR + CP grup, HE, Bar: 50 μ m, C. Karaciğerde normal histolojik görünüm, CUR grup, HE, Bar: 50 μ m, D. Kontrol grubunda karaciğerin normal histolojik görünümü, HE, Bar: 50 μ m

CP administration caused significant increases in MDA levels and decreases in SOD levels in the liver and kidneys when compared with the control and other treated groups. However, the CUR+CP-treated group in all tissues had lower MDA levels and higher SOD levels compared to the SLY+CP group, but the differences were not significant.

Levels of the tissues' DNA fragmentation in the CP-

treated group were higher than those found in the control group and other treated groups, and the difference was statistically significant ($P < 0.001$). The values of DNA fragmentation levels of the kidney tissues in the SLY+CP- and CUR+CP-treated groups were significantly higher than those in the control group. However, no statistically significant differences were observed in the values of the DNA fragmentation of the liver in the SLY+CP- and CUR+CP-treated groups compared to the control group.

Table 2. The degree of histopathological findings in liver and kidney tissues**Tablo 2.** Karaciğer ve böbrekte histopatolojik bulguların dağılımı

Histopathological Findings	Experimental Groups						
	Control	CP	SLY	CUR	SLY+ CP	CUR+ CP	P
Liver							
Disorganization of hepatic cord	00.00±0.00 ^c	3.00±0.00 ^a	00.00±0.00 ^c	00.00±0.00 ^c	1.83±0.30 ^b	1.66±0.21 ^b	***
Sinusoidal dilatation	00.00±0.00 ^c	2.83±0.16 ^a	00.00±0.00 ^c	00.00±0.00 ^c	1.66±0.21 ^b	1.50±0.22 ^b	***
Hyperemia	00.00±0.00 ^c	2.83±0.16 ^a	00.00±0.00 ^c	00.00±0.00 ^c	1.66±0.21 ^b	1.50±0.22 ^b	***
Haemorrhage	00.00±0.00 ^c	1.00±0.25 ^a	00.00±0.00 ^c	00.00±0.00 ^c	0.16±0.16 ^b	0.16±0.16 ^b	***
Degeneration	00.00±0.00 ^c	3.00±0.00 ^a	00.00±0.00 ^c	00.00±0.00 ^c	1.83±0.30 ^b	1.66±0.21 ^b	***
Single cell necrosis	00.00±0.00 ^c	2.83±0.16 ^a	00.00±0.00 ^c	00.00±0.00 ^c	1.66±0.21 ^b	1.33±0.21 ^b	***
Nuclear changes	00.00±0.00 ^c	2.83±0.16 ^a	00.00±0.00 ^c	00.00±0.00 ^c	1.66±0.21 ^b	1.50±0.22 ^b	***
Lipid degeneration	00.00±0.00 ^c	3.00±0.00 ^a	00.00±0.00 ^c	00.00±0.00 ^c	1.50±0.22 ^b	1.16±0.16 ^b	***
Bile duct hyperplasia	00.00±0.00 ^c	2.33±0.33 ^a	00.00±0.00 ^c	00.00±0.00 ^c	1.33±0.21 ^b	1.16±0.16 ^b	***
Mononuclear cell infiltration	00.00±0.00 ^b	1.00±0.25 ^a	00.00±0.00 ^b	00.00±0.00 ^b	0.16±0.16 ^b	0.16±0.16 ^b	***
Kidney							
Oedema	00.00±0.00 ^c	2.66±0.21 ^a	00.00±0.00 ^c	00.00±0.00 ^c	1.66±0.21 ^b	1.16±0.16 ^b	***
Glomerular congestion	00.00±0.00 ^c	2.66±0.21 ^a	00.00±0.00 ^c	00.00±0.00 ^c	1.66±0.21 ^b	1.16±0.16 ^b	***
Interstitial haemorrhage	00.00±0.00 ^c	2.66±0.21 ^a	00.00±0.00 ^c	00.00±0.00 ^c	1.00±0.00 ^b	0.50±0.22 ^b	***
Tubular dilatation	00.00±0.00 ^c	2.83±0.16 ^a	00.00±0.00 ^c	00.00±0.00 ^c	1.66±0.21 ^b	1.33±0.21 ^b	***
Tubular degeneration and desquamation	00.00±0.00 ^c	2.83±0.16 ^a	00.00±0.00 ^c	00.00±0.00 ^c	1.50±0.22 ^b	1.16±0.16 ^b	***
Tubular necrosis	00.00±0.00 ^c	2.83±0.16 ^a	00.00±0.00 ^c	00.00±0.00 ^c	1.50±0.22 ^b	1.33±0.21 ^b	***
Intraluminal hyaline cast	00.00±0.00 ^c	2.50±0.22 ^a	00.00±0.00 ^c	00.00±0.00 ^c	1.50±0.22 ^b	1.33±0.21 ^b	***
Mononuclear cell infiltration	00.00±0.00 ^b	1.33±0.21 ^a	00.00±0.00 ^c	00.00±0.00 ^c	0.33±0.21 ^b	0.16±0.16 ^b	*

^{a, b, c, d} Different letters indicate statistically significant differences in the same row, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$; CP: Cyclophosphamide, SLY: Silymarin, CUR: Curcumin

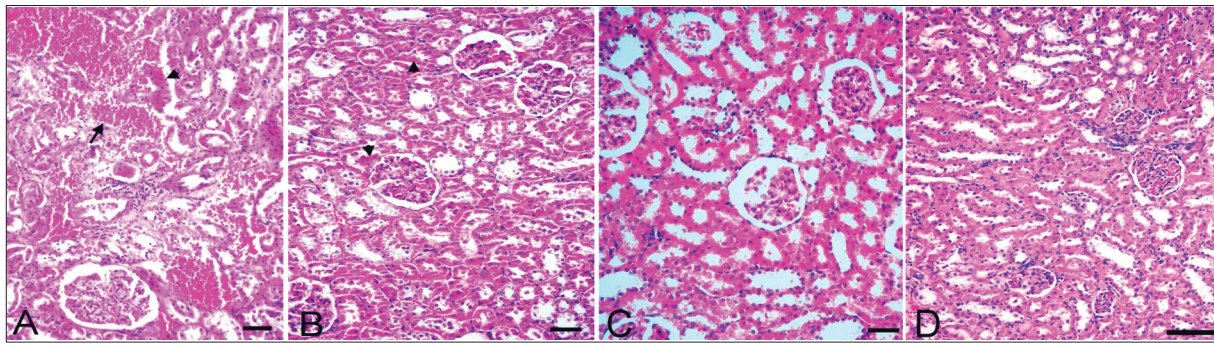


Fig 2. Kidney. A. Severe interstitial haemorrhage (arrow) and severe necrosis in the tubular epithelial cells (arrowhead), CP group, HE, Bar: 50 μ m, B. Mild necrosis in the epithelia of the tubules (arrowheads) SLY + CP group, HE, Bar: 50 μ m, C. Normal histological appearance of kidney, SLY group, HE, Bar: 50 μ m, D. Histological appearance of kidney in control group. HE, Bar: 80 μ m

Şekil 2. Böbrek. A. Şiddetli kanama (ok) ve tubulus epitellerinde şiddetli nekroz (okbaşı), CP grup, HE, Bar: 50 μ m, B. Tubulus epitellerinde hafif nekrozlar (okbaşları), SLY + CP grup, HE, Bar: 50 μ m, C. Böbrekte normal histolojik görünüm, SLY grup, HE, Bar: 50 μ m, D. Kontrol grubunda böbreğin normal histolojik görünümü, HE, Bar: 80 μ m

Histopathological Findings

Microscopic findings of the liver and kidney were summarized in Table 2. In all the tissues, normal morphological findings were seen in the control, SLY and CUR groups. Severe histopathological changes in the liver and kidneys were consistently observed in the CP-treated

group. Histopathological findings in the SLY+CP- and CUR+CP-treated groups were similar to those in the CP-treated group, but there was a prominent decrease in the frequency of pathological results.

In the liver, central veins and sinusoids were dilated. Focal haemorrhages were also noted. Disorganisation

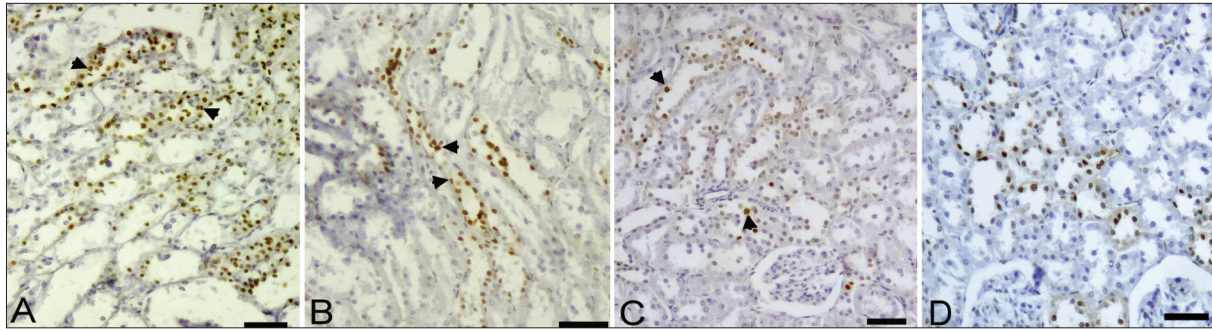


Fig 3. Kidney. **A.** A significant increases in the percentage of the TUNEL positive cells (*arrowheads*), CP Group, TUNEL, Bar: 50 μ m, **B.** A decreases in the percentage of the TUNEL positive cells (*arrowheads*), CUR + CP Group, TUNEL, Bar: 50 μ m, **C.** The percentage of the TUNEL positive cells (*arrowheads*) in the CUR group. CUR group, TUNEL, Bar: 50 μ m, **D.** The percentage of the TUNEL positive cells in the Control group, TUNEL, Bar: 50 μ m

Şekil 3. Böbrek. **A.** TUNEL pozitif hücrelerin oranında artışlar (*okbaşları*), CP Grup, TUNEL, Bar: 50 μ m, **B.** TUNEL pozitif hücrelerin oranında azalma (*okbaşları*), CUR + CP Grup, TUNEL, Bar: 50 μ m, **C.** CUR grubunda TUNEL pozitif hücreler (*okbaşları*), CUR grubu, TUNEL, Bar: 50 μ m, **D.** Kontrol grubunda TUNEL pozitif hücreler, Kontrol grubu, TUNEL, Bar: 50 μ m

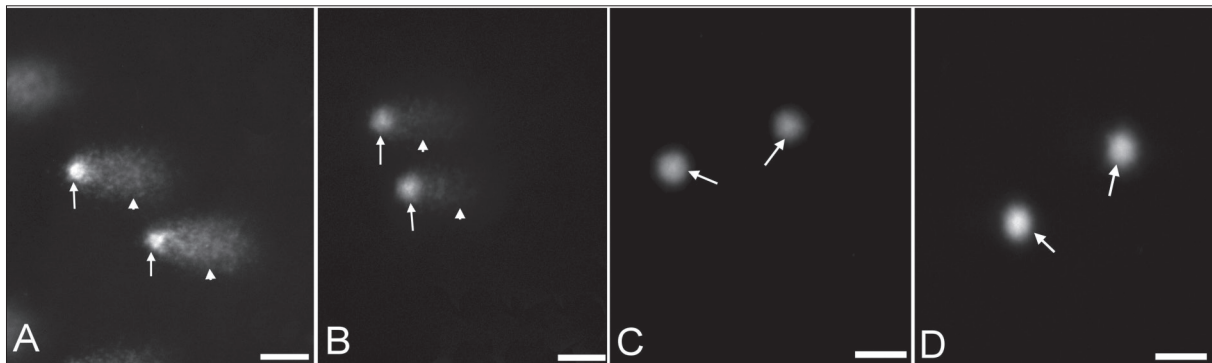


Fig 4. The fluorescence microscopy images of lymphocytes. **A.** CP group, Bar: 30 μ m, **B.** CUR + CP group, Bar: 30 μ m, **C.** CUR group, Bar: 30 μ m, **D.** Control group, Bar: 30 μ m; *Arrows:* head of lymphocytes, *arrowheads:* tail migration of lymphocytes

Şekil 4. Lenfositlerin floresan mikroskopik incelenmesi. **A.** CP grup, Bar: 30 μ m, **B.** CUR + CP grup, Bar: 30 μ m, **C.** CUR grup, Bar: 30 μ m, **D.** Kontrol grubu, Bar: 30 μ m; *oklar:* lenfosit, *okbaşları:* lenfosit kuyruk yoğunluğu

of the hepatic cord, hydropic degeneration and lipid degeneration were common histopathological findings. These findings were generally more pronounced in the sentroaciner areas. Most hepatocytes in the degenerative areas were so swollen, and several hepatocytes had ruptured. Hepatocytes with eosinophilic cytoplasm with picnotic nuclei were also detected in the same areas (*Fig. 1*). In some hepatocytes, the nuclei were swollen, and enlarged, and in some cells they were a few times larger than normal. However, many hepatocytes had moderate to severe cytoplasmic vacuolation, indicating that fatty changes. These fatty droplets were confirmed with Oil Red O. In addition, mononuclear cell infiltrations was seen in portal areas. In kidney tissues, haemorrhages appeared mostly in the cortex (*Fig. 2*). In areas with severe degeneration, cellular integrity was disrupted in many tubular epithelia, and the cytoplasm of intact epithelia had a granular appearance. Nuclei had a picnotic appearance, and the cytoplasm was eosinophilic in epithelia with necrotic changes. In the lumens of many tubules, eosinophilic hyalin casts of varying sizes and irregular structures were present in

some regions, these casts filled in the lumens of tubules completely.

TUNEL assay

The number of cells undergoing apoptosis in the liver and kidneys was determined by a TUNEL assay. The percentage of TUNEL-positive cells per total cells in the liver and kidney (*Fig. 3*) tissues was evaluated and is presented in *Table 1*. A significant increase in the percentage of TUNEL-positive cells was detected in the CP-treated group compared to the control and other treated groups. However, there were significant differences in the percentage of TUNEL-positive cells in the SLY+CP- and CUR+CP-treated groups compared to the control group. The percentage of TUNEL-positive cells in all the tissues of the CUR+CP-treated groups was lower than that of the SLY+CP-treated groups, but the differences did not reach statistical significance.

Comet Assay

Comet-assay examination revealed that the CP administrated group showed a statistically significant

increase in the olive tail moment and the percentage of DNA in the tail levels compared to the control and other treated groups (Fig. 4). The statistical differences of comet assay parameters in all groups are summarised in Table 1. However, the comet parameter levels in the SLY+CP- and CUR+CP-treated groups were lower than those in the CP-treated group, and these values reached the levels of the control and SLY- and CUR-treated groups.

DISCUSSION

CP is an alkylating agent widely used in cancer chemotherapy, and its cytotoxic effects are the result of chemically reactive metabolites that alkylate DNA and protein, producing cross-links [1,2]. The major limitation of using CP is injury to normal tissues, which gives rise to numerous side effects [25]. It has been reported that oxidative-stress-mediated disruption of redox balance after CP exposure generates biochemical and physiological disturbances [5,7]. To date, a number of studies have reported that CP has a prooxidant character and that generation of oxidative stress after CP administration leads to a decrease in the activities of antioxidant systems and increases in lipid peroxidation in some tissues of mice and rats [5]. In both human and veterinary medicine, when tumours are resistant to antineoplastic drugs or when the doses of such drugs are inadequate, many drugs used in chemotherapy, including CP, should be administered at high doses [26]. The most important concern regarding high CP doses is the drug's toxic effects on the tissues [27,28]. It has been reported that CP's toxic effect is mediated by the degradation of antioxidant defence systems by acrolein, which is formed by the metabolism of CP, resulting in excessive production of free radicals [2,3]. In the present study, SLY and CUR, which have strong antioxidant characteristics, were chosen in order to make it possible to use CP in high doses.

Although oxidative stress plays a part in many pathological cycles in the body, excessive amounts of free radicals arising in these processes are counterbalanced by the antioxidant system; if this balance is not maintained, tissue injury occurs [29,30]. It was reported that these radicals lead to peroxidation and modification by oxidising carbohydrates, lipids, proteins and DNA in the cell, exerting a quite toxic effect [29]. One of the reactive metabolites formed during lipid peroxidation, associated with the reactions of free radicals, is MDA [29]. In this study, tissue MDA levels were significantly higher in the CP-only group, indicating that CP increased lipid peroxidation and induced oxidative stress significantly, as reported in the literature [7,30]. However, it has been suggested that lipid peroxidation might be a contributing factor to the development of hepatic and renal toxicity. It is likely that the actions of SLY and CUR in reducing the membrane damage is partially related to their ability to scavenge lipid peroxidation initiating agents [7,8,30,31].

It has been reported by many investigators that SLY [6,12,31] and CUR [7,8,16] have protective activity against oxidative stress caused by various chemical agents. In the present study, MDA levels were found to be significantly lower in the tissues of rats administered SLY and CUR concurrently with CP than in the tissues of rats administered CP by itself, which is consistent with the literature. Although it was established that CUR, by preventing lipid peroxidation, decreased tissue MDA levels more than SLY did, the difference was not statistically significant. SOD enzyme are among the most important defence mechanisms against the damage caused by free oxygen radicals, and it has been reported that toxic superoxide radicals play an important role in the defence against harmful effects by accelerating dismutation to hydrogen peroxide and molecular oxygen [18]. Many studies have investigated the effects of SLY and CUR on antioxidant enzymes in various tissues [30,32]. In the present study, SOD enzyme activity levels were found to be significantly lower than those in the control group in the CP-only group, which suggests that CP may markedly induce superoxide anion radical formation [5]. In the present study, SOD enzyme activity levels in the groups in which SLY and CUR were administered concurrently with CP, were found to be significantly different from those in the CP-only group, suggesting that both SLY and CUR influence the antioxidant system via SOD in the liver and kidneys, as in other chemical agents reported in the literature [4,8].

Serum ALT and AST values have been reported to be influenced by chemical agents having a toxic effect on hepatocytes in the liver, and ALT is considered a more important parameter than AST [33]. Creatinine is known to be an important biochemical parameter indicating kidney function disorders [34]. In the present study, in the CP-only group, liver ALT and AST levels and kidney creatinine levels were found to be significantly higher than those in the other groups, as reported in other studies [3,34]. However, in the histopathological examination of liver and kidney tissues in the present study, pathological findings were more severe in the CP-only group, indicating that histomorphological changes should also be used in the evaluation of these parameters when they are applied to liver and kidney tissues, which is consistent with previous reports [32,34]. In the groups in which SLY and CUR were administered concurrently with CP, ALT levels were found to be significantly lower than those in the CP-only group, suggesting that both SLY and CUR may affect serum ALT levels. Serum AST levels were found to be significantly lower in the groups in which SLY and CUR were administered concomitantly with CP than in the CP-only group. Nevertheless, because there was a significant difference between these groups and the control group in terms of AST level, we speculated that the impact of SLY and CUR on these groups may be weak. In the present study, serum kidney creatinine levels were also significantly lower in the groups in which SLY and CUR were administered

concurrently with CP than in the CP-only group, indicating that SLY and CUR affected kidney creatinine levels, but the difference between them and the control group was not statistically significant.

In the present study, the liver and kidneys of rats in all groups were examined microscopically and macroscopically, and the most common and severe findings were in the CP-only group. These findings are compatible with those of many studies aiming to decrease the toxic effect of CP [3,7,35]. In the present study, there was seen a decreasing in the severity of histopathological changes in the liver and kidney tissue of rats in the groups in which SLY and CUR were administered concurrently with CP than in the CP-only group, demonstrating that these two antioxidants have protective characteristics at the histomorphological level. Our results suggest that the protective mechanism of SLY and CUR might be due to the strong antioxidant property i.e. they helped for healing of liver and kidney parenchyma and regeneration of hepatocytes and tubular epithelial cells.

In order to determine apoptosis in tissues, various techniques, including morphological, immunohistochemical, biochemical, immunological and molecular biology methods, are employed [36,37]. In the present study, in order to measure apoptotic DNA breakages in living tissue, ELISA was chosen from among immunological methods [37], and for use in paraffin sections, TUNEL was chosen from among immunohistochemical methods [37]. According to the ELISA and TUNEL results, the rate of apoptotic cells was significantly higher in the tissues of rats to which CP only was administered, as reported in the literature [38], indicating that high doses of CP are quite effective on apoptosis in tissues. In addition, significantly lower rates of apoptosis were found in the groups in which SLY and CUR were administered together with CP than in the CP-only group, which suggests that both antioxidants decrease the rate of apoptosis caused by the toxic effects of CP.

Single-cell gel electrophoresis or the comet method is a noninvasive, rapid and sensitive fluorescent microscopic method used to determine the rate of DNA damage at the cellular level [21,22]. It has been reported that in the quantitative determination of DNA damage using the comet method, olive tail moment and the percent tail DNA are the most commonly used parameters [21]. In the present study, as in previous research, olive tail moment and the percent tail DNA were used as indicators of DNA damage [22,23]. The significant increase in the aforementioned parameters in the CP-only group compared to the groups in which CP was administered together with SLY and CUR suggests that CP has a marked genotoxic effect on DNA, and it was concluded that both SLY and CUR may be used reliably against the genotoxic effect of CP in cases where it has to be used in high doses. In addition, the lack of a statistically significant difference between the values in these groups and those in the control group supports this conclusion.

In conclusion, in the present study, the most severe pathological findings were seen in the CP-only group, and all serum and tissue biochemical parameters were influenced adversely in this group, indicating that CP exerted a markedly toxic effect on the tissues examined. In addition, it was observed that the findings obtained with the comet method were higher in the CP-only group than in the other groups. Better biochemical, pathological and genotoxic results were obtained in the groups in which SLY and CUR were administered concurrently with CP; this suggests that these antioxidants can be used in conditions when CP has to be administered at high doses. The present study established that both SLY and CUR are effective against the toxic effects of high doses of CP.

REFERENCES

- Dollery C:** Cyclophosphamide. In, Dollery C (Ed): Therapeutic Drugs. 349-354, Churchill Livingstone, Edinburg, 1999.
- McCarroll N, Keshava N, Cimino M, Chu M, Dearfield K, Keshava C, Kligerman A, Owen R, Protzel A, Putzrath R, Schoeny R:** An evaluation of the mode of action framework for mutagenic carcinogens case study: Cyclophosphamide. *Environ Mol Mutagen*, 49, 117-131, 2008. DOI: 10.1002/em.20372
- Senthilkumar S, Yogeeta SK, Subashini R, Devaki T:** Attenuation of cyclophosphamide induced toxicity by squalene in experimental rats. *Chem Biol Interact*, 160, 225-260, 2006. DOI: 10.1016/j.cbi.2006.02.004
- Bjelogrlic SK, Radic J, Jovic V, Radulovic S:** Activity of d,l- α -tocopherol (vitamin E) against cardiotoxicity induced by doxorubicin and doxorubicin with cyclophosphamide in mice. *Basic Clin Pharmacol Toxicol*, 97, 311-319, 2005. DOI: 10.1111/j.1742-7843.2005.pto_166.x
- Ceribaşı AO, Turk G, Sonmez M, Sakin F, Ateşşahin A:** Toxic effect of cyclophosphamide on sperm morphology, testicular histology and blood oxidant-antioxidant balance, and protective roles of lycopene and ellagic acid. *Basic Clin Pharmacol Toxicol*, 107, 730-736, 2010. DOI: 10.1111/j.1742-7843.2010.00571.x
- Kren V, Walterova D:** Silybin and silymarin-new effects and applications. *Biomed Papers*, 149, 29-41, 2005.
- Arafa HM:** Uroprotective effects of curcumin in cyclophosphamide-induced haemorrhagic cystitis paradigm. *Basic Clin Pharmacol Toxicol*, 104, 393-399, 2009. DOI: 10.1111/j.1742-7843.2009.00379.x
- Garcia-Nino WR, Pedraza-Chaverri J:** Protective effect of curcumin against heavy metals-induced liver damage. *Food Chem Toxicol*, 69, 182-201, 2014. DOI: 10.1016/j.fct.2014.04.016
- Hamid AA, Aiyelaagbe OO, Usman LA, Ameen OM, Lawal A:** Antioxidants: Its medicinal and pharmacological applications. *Afr J Pure Appl Chem*, 48, 142-151, 2010.
- Ding TM, Tian SJ, Zhang ZX, Gu DZ, Chen YF, Shi YH, Sun ZP:** Determination of active component in silymarin by RP-LC and LC/MS. *J Pharm Biomed Anal*, 26, 155-61, 2001. DOI:10.1016/S0731-7085(01)00364-8.
- Flora K, Hahn M, Rosen H, Benner K:** Milk thistle (*Silybum marianum*) for the therapy of liver disease. *Am J Gastroenterol*, 93, 139-143, 1998.
- Radko L, Cybulski W:** Application of silymarin in human and animal medicine. *J Pre Clin Clin Res*, 1, 22-26, 2007.
- Sharma RA, Gescher AJ, Steward WP:** Curcumin: The story so far. *Eur J Cancer*, 41, 1955-1968, 2005. DOI: 10.1016/j.ejca.2005.05.009
- Saller R, Meier R, Brignoli R:** The use of silymarin in the treatment of liver diseases. *Drugs*, 61, 2035-2063, 2001.
- Surai PF:** Silymarin as a natural antioxidant: An overview of the current evidence and perspectives. *Antioxidants*, 4, 204-247, 2015. DOI: 10.3390/antiox4010204
- Akram M, Uddin S, Ahmed A, Usmanghani K, Hannan A, Mohiuddin**

- E, Asif M:** *Curcuma longa* and curcumin: A review article. *Rom J Biol*, 55, 65-70, 2010.
- 17. Placer ZA, Cushman LL, Johnson BC:** Estimation of products of lipid peroxidation (malonyldialdehyde) in biochemical systems. *Anal Biochem*, 16, 359-364, 1966. DOI: 10.1016/0003-2697(66)90167-9
- 18. Sun Y, Oberley LW, Li Y:** A simple method for clinical assay of superoxide dismutase. *Clin Chem*, 34, 497-500, 1988.
- 19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ:** Protein measurement with the folin phenol reagent. *J Biol Chem*, 193, 265-275, 1951.
- 20. Yüce A, Ateşşahin A, Çeribaşı AO:** Amelioration of cyclosporine a-induced renal, hepatic and cardiac damages by ellagic acid in rats. *Basic Clin Pharmacol Toxicol*, 103, 186-191, 2008. DOI: 10.1111/j.1742-7843.2008.00284.x
- 21. Collins AR:** The comet assay for DNA damage and repair: Principles, applications, and limitations. *Mol Biotechnol*, 26, 249-61, 2004.
- 22. Singh NP, McCoy MT, Tice RR, Schneider EL:** A Simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res*, 175, 184-191, 1988. DOI:10.1016/0014-4827(88)90265-0
- 23. Boyacioglu M, Sekkin S, Kum C, Korkmaz D, Kiral F, Yalinkilic HS, Ak MO, Akar F:** The protective effects of vitamin C on the DNA damage, antioxidant defenses and aorta histopathology in chronic hyperhomocysteinemia induced rats. *Exp Toxicol Pathol*, 66, 407-413, 2014. DOI: 10.1016/j.etp.2014.06.004
- 24. Conover WJ:** Practical Nonparametric Statistics. John Wiley&Sons, 229-239, New York 1980.
- 25. Fraiser LH, Kanekel S, Kehrer JP:** Cyclophosphamide toxicity. Characterising and avoiding the problem. *Drugs*, 42, 781-795, 1991.
- 26. Cavalletti E, Tofanetti O, Zunino F:** Comprasion of reduced glutathione with 2-mercaptoethane sulfonate to prevent cyclophosphamide-induced urotoxicity. *Cancer Lett*, 32, 1-6, 1986. DOI: 10.1016/0304-3835(86)90032-7
- 27. Schimmel KJ, Richel DJ, Brink RBAVD, Guchelaar HJ:** Cardiotoxicity of cytotoxic drugs. *Cancer Treat Rev*, 30, 181-191, 2004. DOI: 10.1016/j.ctrv.2003.07.003
- 28. Xu X, Malave A:** Protective effect of berberine on cyclophosphamide-induced haemorrhagic cystitis in rats. *Pharmacol Toxicology*, 88, 232-377, 2001.
- 29. Halliwell B, Gutteridge JM:** Lipid peroxidation, oxygen radicals, cell damage, and antioxidant therapy. *Lancet*, 23, 1396-1397, 1984.
- 30. Taghiabadi E, Imenshahidi M, Abnous K, Mosafa F, Sankian M, Memar B, Karimi G:** Protective effect of silymarin against acrolein-induced cardiotoxicity in mice. *J Evid Based Complementary Altern Med*, 2012 (2012), Article ID 352091, 2012. DOI: 10.1155/2012/352091
- 31. Rastogi R, Srivastava AK, Srivastava M, Rastogi AK:** Hepatocurative effect of picroliv and silymarin against aflatoxin B1 induced hepatotoxicity in rats. *Planta Med*, 66, 709-713, 2000. DOI: 10.1055/s-2000-9907
- 32. Ahmida MH:** Protective role of curcumin in nephrotoxic oxidative damage induced by vancomycin in rats. *Exp Toxicol Pathol*, 64, 149-53, 2012. DOI: 10.1016/j.etp.2010.07.010
- 33. Ozer J, Ratner M, Shaw M, Bailey W, Schomaker S:** The current state of serum biomarkers of hepatotoxicity. *Toxicology*, 245, 194-205, 2008. DOI: 10.1016/j.tox.2007.11.021
- 34. Alhumaidha KA, Saleh DO, Abd El Fattah MA, El-Eraky WI, Moawad H:** Cardiorenal protective effect of taurine against cyclophosphamide-induced toxicity in albino rats. *Can J Physiol Pharmacol*, 18, 131-139, 2016. DOI: 10.1139/cjpp-2015-0138
- 35. Motawi TM, Sadik NA, Refaat A:** Cytoprotective effects of DLalpha-lipoic acid or squalene on cyclophosphamide-induced oxidative injury: An experimental study on rat myocardium, testicles and urinary bladder. *Food Chem Toxicol*, 48, 2326-36, 2010. DOI: 10.1016/j.fct.2010.05.067
- 36. Elmore S:** Apoptosis: A review of programmed cell death. *Toxicol Pathol*, 35, 495-516, 2007. DOI: 10.1080/01926230701320337
- 37. Basha WM, Ahmed MI, Hafez RAEL, El-Bialy AAAEK, Amer A:** Prognostic value of bcl-2, bcl-xl and bax expression and serum DNA fragmentation products in patients with primary laryngeal carcinomas. *Egypt J Med Microbiol*, 19, 9-22, 2010.
- 38. Saandeep K, Vikram A, Tripathi DN, Ramarao P, Jena, G:** Influence of hyperglycaemia on chemical-induced toxicity: Study with cyclophosphamide in rat. *Basic Clin Pharmacol Toxicol*, 105, 236-242, 2009. DOI: 10.1111/j.1742-7843.2009.00433.x