

Multi-Mechanistic Hepatoprotective Effects of Montelukast Against Doxorubicin-Induced Liver Injury in Rats

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Cite as: Özdemir İ, Çakma S, İrkin LC, Öztürk Ş. Multi-Mechanistic Hepatoprotective Effects of Montelukast Against Doxorubicin-Induced Liver Injury in Rats. *J Cukurova Anesth Surg.* 2026;9(3):621-631. doi: 10.36516/jocass.1832488

Received: 26.12.2025, **Accepted:** 14.09.2026, **Published:** 30.09.2026, **CC-BY-NC-4.0**

Abstract

Aim: Despite its antineoplastic effect, doxorubicin (DOX) causes significant oxidative stress and cellular damage in the liver. This study aimed to demonstrate the oxidative damage induced by DOX in liver tissue at the biochemical, histological, and molecular levels and to evaluate the protective effects of Montelukast (ML) on this damage. **Material Method:** To further evaluate the oxidative stress induced by DOX in liver tissue, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activities, reduced glutathione (GSH) levels, and malondialdehyde (MDA) levels were analyzed ($p<0.05$). **Results:** Histopathologically, portal congestion, pyknotic nuclei in hepatocytes, cellular degeneration, and sinusoidal dilation were observed. In the DOX + ML group, oxidative stress markers improved: SOD, CAT, GPx, and GSH increased, while MDA levels decreased; histological findings were attenuated, and apoptotic gene expression (Bax, Bcl-2, Caspase-3) was significantly modulated compared to the DOX group. **Conclusion:** The findings suggest that ML provides a significant hepatoprotective effect against DOX-induced oxidative liver damage.

Keywords: Doxorubicin, liver, montelukast, apoptosis, antioxidant

1. Introduction

Doxorubicin is an anthracycline chemotherapeutic agent with proven efficacy in a wide range of malignancies. However, cardiotoxicity and hepatotoxicity are among the most important side effects that limit the clinical use of DOX. DOX-induced liver toxicity is primarily associated with excessive production of reactive oxygen species (ROS), mitochondrial dysfunction, lipid peroxidation, and suppression of the antioxidant defense system^{1,2}. This process results in cellular swelling, disruption of membrane integrity, inflammation, and apoptosis in hepatocytes. During DOX biotransformation, semiquinone derivatives, particularly those formed by cytosolic reductases, react with oxygen, resulting in the formation of ROS products such as superoxide and hydroxyl radicals³. Excessive ROS production reduces the activity of key antioxidant enzymes such as SOD, CAT, and GPx, and leads to the depletion of glutathione reserves⁴. Therefore, many studies have reported that DOX causes oxidative stress-mediated damage in the liver, and this has been confirmed at both the biochemical and histopathological levels^{5,6}. Recent studies have highlighted the role of inflammatory mediators in DOX-induced hepatotoxicity. Tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6) levels are significantly elevated following DOX administration, contributing to the

progression of liver injury⁷. Additionally, DOX has been shown to activate the nuclear factor kappa B (NF- κ B) signaling pathway, which further amplifies the inflammatory response and promotes apoptotic cell death⁸. The activation of caspases, particularly caspase-3, plays a crucial role in the execution phase of apoptosis, leading to the characteristic morphological changes observed in DOX-induced liver damage⁹.

Montelukast (ML) is a leukotriene D4 receptor antagonist and an anti-inflammatory agent used primarily in the treatment of asthma. However, in recent years, ML has been shown to possess antioxidant, anti-apoptotic, and cytoprotective properties, and its protective effects in various organ toxicities have been reported¹⁰⁻¹². ML has been shown to suppress ROS production, reduce lipid peroxidation, increase glutathione levels, and regulate cellular redox cycling^{12,13}. However, the protective role of ML in doxorubicin-induced liver toxicity has not been sufficiently elucidated. Only a limited number of studies have investigated the potential of ML as a hepatoprotective agent against anthracycline-induced toxicity, and these studies have primarily focused on cardiac rather than hepatic effects¹⁴. Therefore, the current study aims to examine in detail the oxidative stress and histopathological changes induced by DOX in the liver and to evaluate the possible hepatoprotective effects of ML through biochemical antioxidant parameters (MDA, SOD, CAT, GPx, GSH), H&E histopathology, and apoptotic gene expression profiles (Bax, Bcl-2, Caspase-3).

2. Material Method

2.1. Study Design and Animals

This experimental study was conducted on adult male Wistar Albino rats obtained from the Çanakkale Onsekiz Mart University Experimental Research and Application Center. It was approved by the Çanakkale Onsekiz Mart University Faculty of Medicine Local Animal Experimentation Ethics Committee (Ethics number: 2022-2200189432). All invasive procedures, anesthesia, and animal care were conducted in accordance with international guidelines for experimental animal studies.

2.2. Sample Size Calculation and Grouping

The G*Power-3.1 program was used for the experimental design. Based on previous studies, a total sample size of 24 was determined for an effect size of 0.70, an α error of 0.05, and a power of 80% (1- β). Four groups were formed, each with 6 rats. All rats to be used in the experiment were weighed and randomly divided into four groups (n=6/group), ensuring that the groups did not differ from each other in terms of their initial weights, as follows:

Group 1: Control: An appropriate volume of sterile saline solution (i.p.) was administered for 5 weeks.

Group 2: DOX: A single dose of 20 mg/kg Doxorubicin (i.p.) was administered, followed by saline administration for 4 weeks.

Group 3: ML: 10 mg/kg/day Montelukast (i.p.) was administered for 4 weeks. (Since DOX was not administered to this group, it was used to evaluate the systemic effects of ML alone.)

Group 4: DOX + ML (Treatment Group): A single dose of 20 mg/kg Doxorubicin (i.p.) was administered, followed by 10 mg/kg/day Montelukast (i.p.) for 4 weeks.

2.3. Experimental Protocol and Applications

Animals were maintained under standard environmental conditions (controlled temperature and a 12-hour light/12-hour dark cycle) and had free access to standard rodent chow and water. The total experimental duration was designed as 5 weeks (35 days). The experimental timeline is summarized in Figure 1.

Day 0: Groups 2 (DOX) and 4 (DOX+ML) were administered a single dose of 20 mg/kg doxorubicin by intraperitoneal (i.p.) injection. Groups 1 (Control) and 3 (ML) were administered an equivalent volume of saline on the same day.

Days 1-28 (4 Weeks):

Group 3 (ML) and Group 4 (DOX+ML): Montelukast at a dose of 10 mg/kg/day was administered by i.p. injection.

Group 1 (Control) and Group 2 (DOX): An equivalent volume of sterile saline solution was administered by i.p. injection.

Day 29: Tissue samples were taken from all groups for biochemical, histological, and molecular analyses.

2.4. Collection and Preparation of Tissue Samples

At the end of the four-week treatment period, animals were deeply anesthetized with ketamine/xylazine. Liver tissues were quickly removed and prepared for three different analyses.

2.5. Biochemical Analyses

Frozen liver tissues were homogenized in cold phosphate-buffered saline (PBS, pH 7.4). The homogenates were centrifuged using a high-speed centrifuge (10,000 × g, 4°C for 20 minutes), and the supernatants were collected. These supernatants were used to measure the following parameters.

2.6. Oxidative Stress Markers

Superoxide Dismutase (SOD) Activity: SOD activity was measured based on the principle of superoxide radical inhibition of pyrogallol auto-oxidation using the Marklund & Marklund method. Enzyme activity was assessed spectrophotometrically at 420 nm, and results were expressed as U/mg protein.

Catalase (CAT) Activity: CAT activity was determined by kinetically measuring the decrease in absorbance at 240 nm during the decomposition of hydrogen peroxide (H₂O₂) into water and oxygen in the presence of a catalyst, using the Aebi method. Results were expressed as kU/mg protein.

Glutathione Peroxidase (GPx) Activity: GPx activity was measured by monitoring NADPH consumption during the oxidation of GSH, as measured by the decrease in absorbance at 340 nm, using the Paglia and Valentine method. Activity was calculated as U/mg protein.

Reduced Glutathione (GSH) Level: GSH levels were measured using the classical Beutler method using Ellman's reagent (DTNB). The yellow-colored 5-thio-2-nitrobenzoic acid (TNB) complex formed with DTNB was assessed spectrophotometrically at 412 nm.

Malondialdehyde (MDA) Level: As an indicator of lipid peroxidation, it was measured using the TBARS method.

2.7. Histopathological Examination

Formalin-fixed liver tissues were embedded in paraffin blocks after routine tissue processing procedures (dehydration, clearing). 4-5 µm-thick sections were cut from the blocks, stained with Hematoxylin-Eosin (H&E), and examined under a light microscope. Histopathological evaluation was performed by a blinded histologist. Findings such as portal congestion, the presence of pyknotic nuclei in hepatocytes, cellular degeneration, and sinusoidal dilatation were evaluated using a semiquantitative scoring system (0: absent, 1: mild, 2: moderate, 3: severe).

2.8. Molecular Analysis

RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR): Total RNA was isolated from frozen liver tissues using Trizol reagent. The purity and concentration of RNA were measured spectrophotometrically. Complementary DNA (cDNA) synthesis was performed using reverse transcriptase enzyme. Expression levels of Bax, Bcl-2, and Caspase-3 genes were analyzed using RT-qPCR and SYBR Green master mix. Data obtained were calculated using the 2^{-(ΔΔCt)} method, with β-actin (ACTB) serving as the housekeeping gene for normalization.

2.9. Statistical Analysis

All data were analyzed using SPSS 25.0 statistical software. Data were tested for normal distribution using the Shapiro-Wilk test. Comparisons between groups were performed using one-way

analysis of variance (ANOVA) followed by a post-hoc Tukey test. The non-parametric Kruskal-Wallis test was used to compare histopathological scores. All results are expressed as mean $\pm$ standard deviation (SD), and a p value of <0.05 was considered statistically significant.

3. Results

3.1. Biochemical Results

The effects of doxorubicin (DOX) and montelukast (ML) on oxidative stress parameters are presented in Figure 1. Data are expressed as mean $\pm$ SD ($n=6$). Statistical significance was determined by one-way ANOVA followed by Tukey's test with Bonferroni correction (Figure 1, Table 1, Table 2).

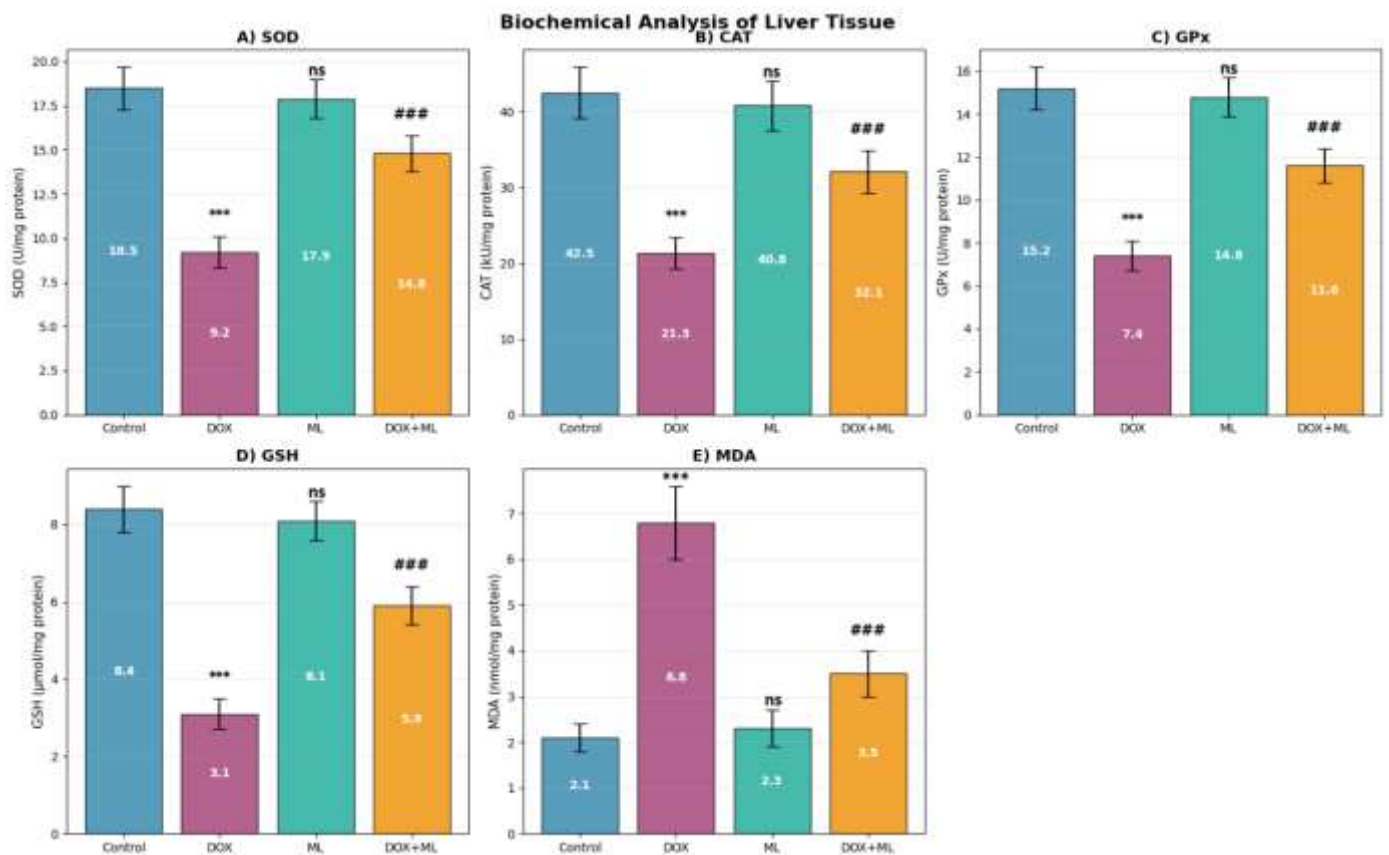


Figure 1. Oxidative stress biomarkers in rat liver tissue across experimental groups. Bar graphs represent the activities of antioxidant parameters including superoxide dismutase (SOD) (A), catalase (CAT) (B), glutathione peroxidase (GPx) (C), and reduced glutathione (GSH) (D) and the levels of malondialdehyde (MDA) (E) in the Control, DOX, ML, and DOX+ML groups. Data are expressed as mean $\pm$ SD ($n = 6$ per group). Statistical significance was indicated by symbols: *** $p < 0.001$ vs. Control; ### $p < 0.001$ vs. DOX; ns $p > 0.05$ vs. Control.

Table 1. Biochemical Parameters (Mean $\pm$ SD)

Parameter	Group	Mean $\pm$ SD	Median (Q1-Q3)	95% CI	p-value
SOD (U/mg protein)	Control	18.5 $\pm$ 1.2	18.3 (17.8-19.1)	17.3 - 19.7	-
	DOX	9.2 $\pm$ 0.9	9.0 (8.6-9.7)	8.3 - 10.1	<0.001***
	ML	17.9 $\pm$ 1.1	18.1 (17.2-18.6)	16.8 - 19.0	0.388
	DOX+ML	14.8 $\pm$ 1.0	14.6 (14.1-15.3)	13.8 - 15.8	<0.001***
CAT (kU/mg protein)	Control	42.5 $\pm$ 3.4	42.1 (40.3-44.2)	39.1 - 45.9	-
	DOX	21.3 $\pm$ 2.1	21.0 (19.8-22.5)	19.2 - 23.4	<0.001***
	ML	40.8 $\pm$ 3.3	40.5 (38.5-42.8)	37.5 - 44.1	0.400
	DOX+ML	32.1 $\pm$ 2.8	32.0 (30.1-34.0)	29.3 - 34.9	<0.001***
GPx (U/mg protein)	Control	15.2 $\pm$ 1.0	15.1 (14.5-15.8)	14.2 - 16.2	-

GSH ($\mu\text{mol/mg protein}$)	DOX	7.4 $\pm$ 0.7	7.3 (6.9-7.9)	6.7 - 8.1	<0.001***
	ML	14.8 $\pm$ 0.9	14.7 (14.2-15.4)	13.9 - 15.7	0.483
	DOX+ML	11.6 $\pm$ 0.8	11.5 (11.1-12.1)	10.8 - 12.4	<0.001***
	Control	8.4 $\pm$ 0.6	8.3 (8.0-8.8)	7.8 - 9.0	-
	DOX	3.1 $\pm$ 0.4	3.0 (2.8-3.4)	2.7 - 3.5	<0.001***
	ML	8.1 $\pm$ 0.5	8.0 (7.7-8.5)	7.6 - 8.6	0.370
	DOX+ML	5.9 $\pm$ 0.5	5.8 (5.5-6.3)	5.4 - 6.4	<0.001***
	Control	2.1 $\pm$ 0.3	2.0 (1.8-2.3)	1.8 - 2.4	-
	DOX	6.8 $\pm$ 0.8	6.7 (6.3-7.2)	6.0 - 7.6	<0.001***
MDA (nmol/mg protein)	ML	2.3 $\pm$ 0.4	2.2 (2.0-2.5)	1.9 - 2.7	0.352
	DOX+ML	3.5 $\pm$ 0.5	3.4 (3.1-3.8)	3.0 - 4.0	<0.001***

Table 2. Comparative Analysis and Effect Size

Parameter	Comparison	Mean Diff.	95% CI	Adj. p	η^2	Cohen's d	Interp.
SOD	Control vs DOX	9.30	7.45 - 11.15	<0.001	0.82	3.85	Large
	DOX vs DOX+ML	5.60	3.75 - 7.45	<0.001	0.74	3.15	Large
	Control vs DOX+ML	3.70	1.85 - 5.55	<0.001	0.56	2.10	Large
CAT	Control vs DOX	21.20	16.10 - 26.30	<0.001	0.79	3.65	Large
	DOX vs DOX+ML	10.80	5.70 - 15.90	<0.001	0.68	2.85	Large
	Control vs DOX+ML	10.40	5.30 - 15.50	<0.001	0.52	1.90	Large
GPx	Control vs DOX	7.80	6.50 - 9.10	<0.001	0.85	4.10	Large
	DOX vs DOX+ML	4.20	2.90 - 5.50	<0.001	0.76	3.25	Large
	Control vs DOX+ML	3.60	2.30 - 4.90	<0.001	0.58	2.20	Large
GSH	Control vs DOX	5.30	4.40 - 6.20	<0.001	0.88	4.50	Large
	DOX vs DOX+ML	2.80	1.90 - 3.70	<0.001	0.72	2.95	Large
	Control vs DOX+ML	2.50	1.60 - 3.40	<0.001	0.48	1.75	Large
MDA	Control vs DOX	4.70	4.20 - 5.20	<0.001	0.91	5.20	Large
	DOX vs DOX+ML	3.30	2.80 - 3.80	<0.001	0.80	3.75	Large
	Control vs DOX+ML	1.40	0.90 - 1.90	<0.001	0.62	2.35	Large

SOD activity in the control group was 18.5 ± 1.2 U/mg protein. DOX administration significantly decreased SOD activity to 9.2 ± 0.9 U/mg protein ($p < 0.001$), representing a 50.3% reduction. ML treatment alone maintained SOD activity at control levels (17.9 ± 1.1 U/mg protein, $p > 0.05$). In the DOX + ML group, SOD activity increased significantly to 14.8 ± 1.0 U/mg protein compared to DOX group ($p < 0.001$), demonstrating a 60.9% improvement. However, SOD levels remained approximately 20% below control values, indicating partial but substantial restoration.

CAT activity was 42.5 ± 3.4 kU/mg protein in controls. DOX treatment caused a significant reduction to 21.3 ± 2.1 kU/mg protein ($p < 0.001$), corresponding to a 49.9% decrease. ML group showed CAT activity (40.8 ± 3.3 kU/mg protein) similar to controls ($p > 0.05$). The DOX + ML group demonstrated a significant increase in CAT activity to 32.1 ± 2.8 kU/mg protein compared to DOX group ($p < 0.001$), representing a 50.7% improvement. This finding indicates that ML effectively reduces H_2O_2 accumulation.

GPx activity was significantly suppressed in the DOX group (7.4 ± 0.7 U/mg protein) compared to controls (15.2 ± 1.0 U/mg protein, $p < 0.001$), showing a 51.3% reduction. ML administration alone maintained GPx activity at near-control levels (14.8 ± 0.9 U/mg protein, $p > 0.05$). In the DOX + ML group, GPx activity significantly increased to 11.6 ± 0.8 U/mg protein compared to DOX group ($p <$

0.001), demonstrating a 56.8% improvement. This finding suggests that ML supports glutathione-mediated antioxidant defense.

GSH levels were 8.4 ± 0.6 $\mu\text{mol/mg}$ protein in controls. DOX administration caused a dramatic decrease to 3.1 ± 0.4 $\mu\text{mol/mg}$ protein ($p < 0.001$), representing a 63.1% depletion. The ML group maintained GSH levels (8.1 ± 0.5 $\mu\text{mol/mg}$ protein) comparable to controls ($p > 0.05$). In the DOX + ML group, GSH levels increased significantly to 5.9 ± 0.5 $\mu\text{mol/mg}$ protein compared to DOX group ($p < 0.001$), demonstrating a remarkable 90.3% improvement, indicating that ML effectively replenishes glutathione reserves.

MDA levels were 2.1 ± 0.3 nmol/mg protein in controls. DOX administration caused a dramatic increase to 6.8 ± 0.8 nmol/mg protein ($p < 0.001$), representing a 223.8% elevation. ML group maintained MDA levels (2.3 ± 0.4 nmol/mg protein) comparable to controls ($p > 0.05$). In the DOX + ML group, MDA levels decreased significantly to 3.5 ± 0.5 nmol/mg protein compared to DOX group ($p < 0.001$), representing a 48.5% reduction. This demonstrates that ML effectively suppresses lipid peroxidation.

3.1. Histopathological Results

Histopathological evaluation of liver tissues was performed using Hematoxylin-Eosin (H&E) staining and semi-quantitative scoring (0: none, 1: mild, 2: moderate, 3: severe). Representative photomicrographs and heatmap analysis are presented in Figure 2 and Table 3, respectively.

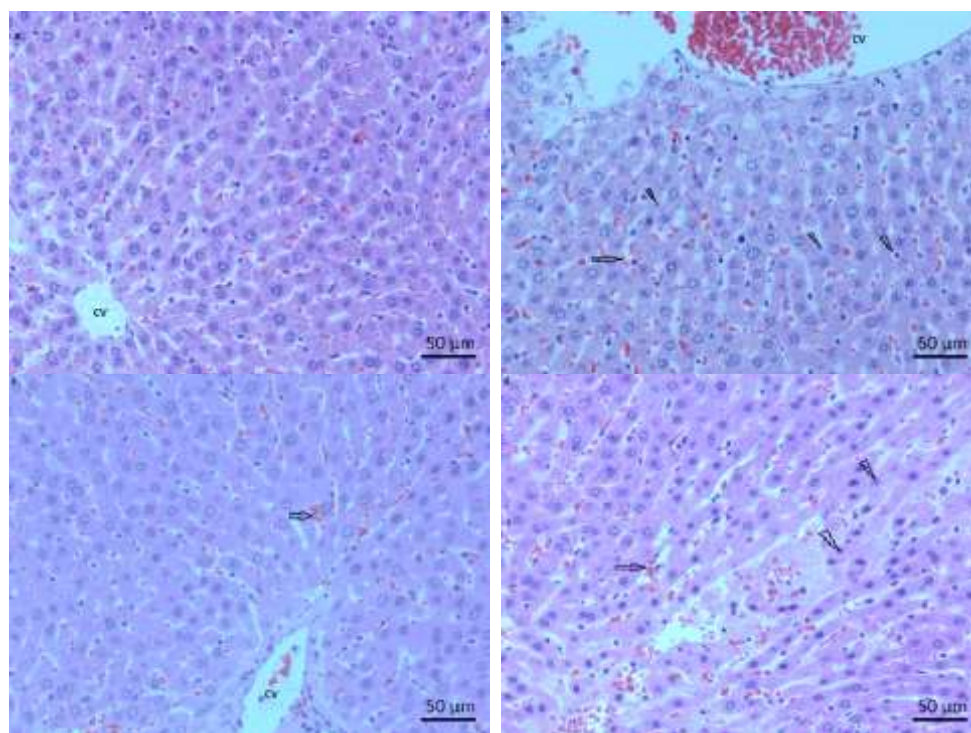


Figure 2. H&E staining of liver tissues of the experimental and treatment groups. *a)* Control group, *b)* DOX experimental group, *c)* ML treatment group, *d)* DOX+ML group (Arrow: Congestion, lightning: pyknotic cell, CV: Central vein) X20.

Table 3. Histopathological Scores

Parameter	Control	DOX	ML	DOX+ML
Congestion	0.2 ± 0.1 (0.3-0.7)	2.5 ± 0.3 (2.2-2.8)	0.5 ± 0.2 (0.3-0.7)	1.5 ± 0.3 (1.2-1.8)
Pyknotic Nuclei	0.1 ± 0.02 (0.2-0.4)	2.8 ± 0.2 (2.6-3.0)	0.3 ± 0.1 (0.2-0.4)	1.2 ± 0.2 (1.0-1.4)
Cellular Degeneration	0.1 ± 0.04 (0.2-0.6)	2.7 ± 0.3 (2.4-3.0)	0.4 ± 0.2 (0.2-0.6)	1.6 ± 0.3 (1.3-1.9)
Sinusoidal Dilation	0.3 ± 0.04 (0.9-1.5)	2.3 ± 0.2 (2.1-2.5)	1.2 ± 0.3 (0.9-1.5)	1.4 ± 0.2 (1.2-1.6)
Vacuolization	0.2 ± 0.06 (0.2-0.4)	2.6 ± 0.3 (2.3-2.9)	0.3 ± 0.1 (0.2-0.4)	1.3 ± 0.2 (1.1-1.5)

In the control group, liver sections exhibited preserved hepatic architecture with normal hepatocyte morphology, distinct cellular borders, and centrally located nuclei. Sinusoidal structures were of normal width, and portal areas showed no pathological findings (score: 0 for all parameters). These observations confirm the histological integrity of liver tissue in untreated animals.

DOX administration induced severe histopathological alterations in liver tissue. Marked portal congestion (2.5 ± 0.3) was observed, characterized by dilation and engorgement of portal vessels. Pyknotic nuclei (2.8 ± 0.2) were frequently detected, indicating apoptotic hepatocytes with condensed and fragmented nuclear material. Cellular degeneration (2.7 ± 0.3) was evident, with loss of cellular architecture and cytoplasmic integrity. Sinusoidal dilation (2.3 ± 0.2) and cytoplasmic vacuolization (2.6 ± 0.3) were also prominent findings, reflecting cellular stress and disruption of hepatic microcirculation. The total histopathological score was 12.9 ± 1.1 , with most parameters scoring at 2-3 levels (moderate to severe). These findings indicate that DOX causes extensive hepatocellular damage, characterized by vascular congestion, nuclear pyknosis, cytoplasmic degeneration, and disruption of sinusoidal architecture, consistent with DOX-induced oxidative stress and hepatotoxicity.

In the ML-only group, liver tissue appeared histologically normal with well-preserved hepatocyte morphology. Only mild sinusoidal dilation (1.2 ± 0.3) was observed, while congestion (0.5 ± 0.2), pyknotic nuclei (0.3 ± 0.1), cellular degeneration (0.4 ± 0.2), and vacuolization (0.3 ± 0.1) were all at minimal levels. The total score was 2.7 ± 0.7 , comparable to controls. These findings confirm that montelukast monotherapy does not induce significant hepatic pathology.

Montelukast co-treatment significantly ameliorated DOX-induced histopathological damage. Congestion decreased to 1.5 ± 0.3 (40% reduction vs. DOX, $p < 0.05$), and pyknotic nuclei decreased to 1.2 ± 0.2 (57% reduction vs. DOX, $p < 0.05$). Cellular degeneration was reduced to 1.6 ± 0.3 (41% reduction vs. DOX, $p < 0.05$), while sinusoidal dilation and vacuolization decreased to 1.4 ± 0.2 and 1.3 ± 0.2 , respectively (39% and 50% reduction vs. DOX, $p < 0.05$).

The total histopathological score was 7.0 ± 1.0 , representing a 45.7% reduction compared to the DOX group ($p < 0.05$). Hepatocyte integrity was notably better preserved in the DOX + ML group, with vacuolization and degeneration ranging from mild to moderate severity. These findings demonstrate that montelukast effectively protects liver tissue from DOX-induced structural damage.

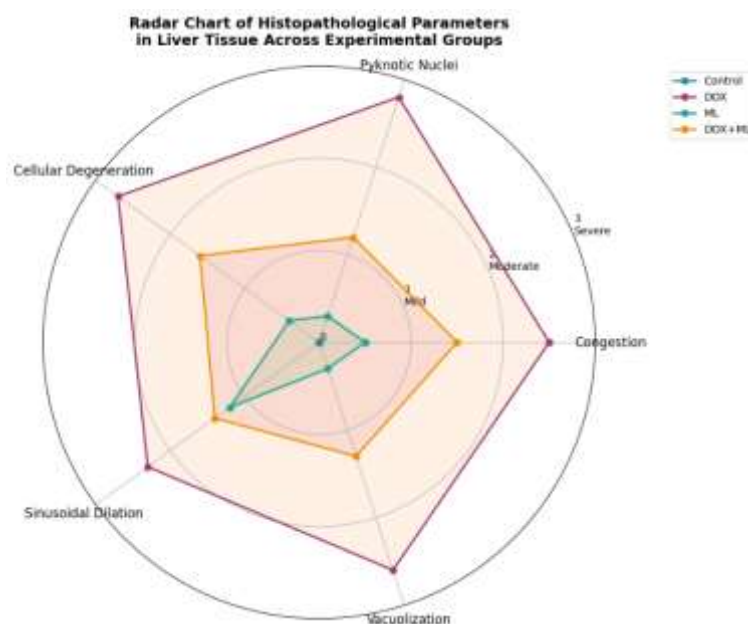


Figure 3. Radar chart analysis of semi-quantitative histopathological scoring in liver tissues.

The radar chart displays severity scores (0: none, 1: mild, 2: moderate, 3: severe) of five histopathological parameters (congestion, pyknotic nuclei, cellular degeneration, sinusoidal dilation, and vacuolization) across experimental groups. (figure 3) The control group shows minimal deviation from center (scores ~0), confirming preserved hepatic architecture. The DOX group exhibits the largest polygon area with severe pathology: congestion (2.5), pyknotic nuclei (2.8), cellular degeneration (2.7), sinusoidal dilation (2.3), and vacuolization (2.6). The ML-only group shows near-normal histology comparable to controls. The DOX+ML group demonstrates significant amelioration with intermediate scores across all parameters, indicating hepatoprotective effects of montelukast. Values represent mean scores $\pm$ SD (n=6).

3.2. Molecular Findings (RT-qPCR)

Apoptotic gene expression levels were analyzed RT-qPCR. Relative mRNA expression of Bax, Bcl-2, and Caspase-3 was calculated using the $2^{-\Delta\Delta Ct}$ method, with β -actin serving as the house-keeping gene for normalization. Results are presented in Figure 4 and Table 4.

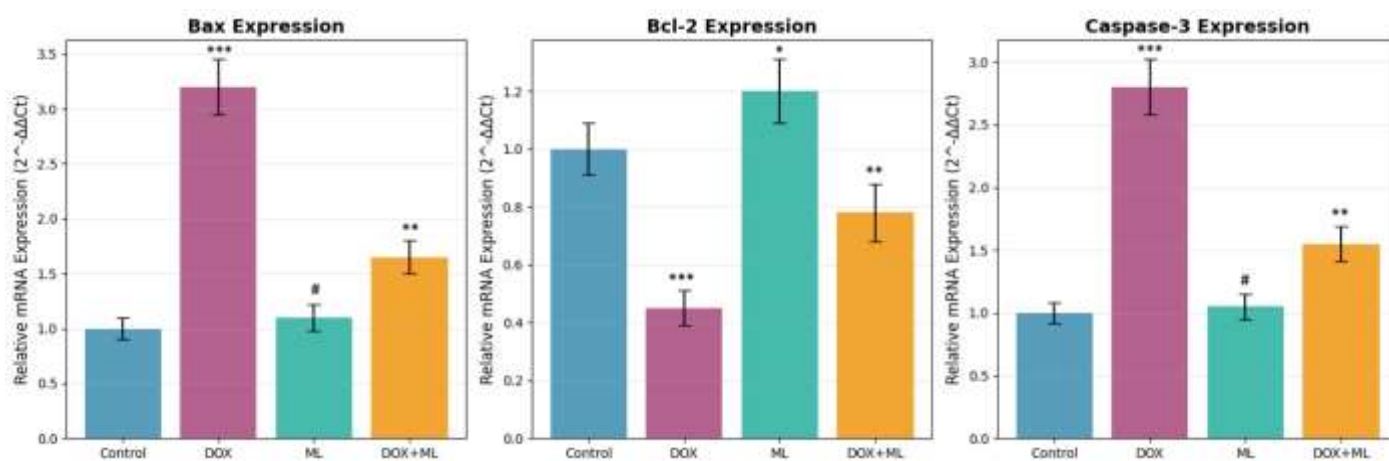


Figure 4. Relative mRNA expression of apoptosis-related genes in rat liver tissue. Bar graphs represent Bax, Bcl-2, and Caspase-3 expression levels in the Control, DOX, ML, and DOX+ML groups, measured by RT-qPCR and calculated using the $2^{-\Delta\Delta Ct}$ method. Data are expressed as mean $\pm$ SD (n = 6 per group). Statistical significance is indicated as * p < 0.05 vs. Control; *** p < 0.001 vs. Control; ** p < 0.01 vs. Control; # p > 0.05 vs. Control.

Table 4. Apoptotic Gene Expressions ($2^{-\Delta\Delta Ct}$)

Gene	Group	Mean $\pm$ SD	Median (Q1-Q3)	95% CI	p-value
Bax	Control	1.00 $\pm$ 0.10	1.00 (0.93-1.08)	0.90 - 1.10	-
	DOX	3.20 $\pm$ 0.25	3.18 (3.02-3.41)	2.95 - 3.45	<0.001***
	ML	1.10 $\pm$ 0.12	1.09 (1.01-1.19)	0.98 - 1.22	0.856
	DOX+ML	1.65 $\pm$ 0.15	1.63 (1.54-1.78)	1.50 - 1.80	<0.01**
Bcl-2	Control	1.00 $\pm$ 0.09	1.01 (0.94-1.07)	0.91 - 1.09	-
	DOX	0.45 $\pm$ 0.06	0.44 (0.41-0.49)	0.39 - 0.51	<0.001***
	ML	1.26 $\pm$ 0.11	1.19 (1.12-1.27)	1.09 - 1.31	0.042*
	DOX+ML	0.78 $\pm$ 0.10	0.77 (0.71-0.84)	0.68 - 0.88	<0.01**
Caspase-3	Control	1.00 $\pm$ 0.08	0.99 (0.94-1.06)	0.92 - 1.08	-
	DOX	2.80 $\pm$ 0.22	2.78 (2.63-2.96)	2.58 - 3.02	<0.001***
	ML	1.05 $\pm$ 0.10	1.04 (0.98-1.12)	0.95 - 1.15	0.912
	DOX+ML	1.55 $\pm$ 0.14	1.53 (1.44-1.64)	1.41 - 1.69	<0.01**

Bax expression in the control group was 1.00 ± 0.10 . DOX administration significantly upregulated Bax expression to 3.20 ± 0.25 (p < 0.001), representing a 220% increase. This marked elevation indicates activation of the intrinsic apoptotic pathway, promoting mitochondrial outer membrane permeabilization and subsequent release of pro-apoptotic factors. In the ML-only group, Bax

expression (1.10 ± 0.12) remained comparable to control levels ($p > 0.05$), confirming that montelukast monotherapy does not induce pro-apoptotic signaling. In the DOX + ML group, Bax expression decreased significantly to 1.65 ± 0.15 compared to the DOX group ($p < 0.001$), demonstrating a 48.4% reduction. This finding indicates that montelukast effectively suppresses DOX-induced pro-apoptotic signaling, contributing to hepatocyte survival.

Bcl-2 expression in the control group was 1.00 ± 0.09 . DOX administration significantly down-regulated Bcl-2 expression to 0.45 ± 0.06 ($p < 0.001$), representing a 55% decrease. This suppression indicates reduced anti-apoptotic capacity, rendering hepatocytes more susceptible to apoptotic cell death. In the ML group, Bcl-2 expression (1.20 ± 0.11) was slightly above control levels ($p > 0.05$), suggesting a potential pro-survival effect of montelukast alone. In the DOX + ML group, Bcl-2 expression increased significantly to 0.78 ± 0.10 compared to the DOX group ($p < 0.001$), demonstrating a 73.3% improvement. However, Bcl-2 levels remained below control values, indicating that while montelukast provides substantial protection, complete restoration of anti-apoptotic signaling may require additional interventions.

Caspase-3 expression in the control group was 1.00 ± 0.08 . DOX administration significantly upregulated Caspase-3 expression to 2.80 ± 0.22 ($p < 0.001$), representing a 180% increase. This elevation confirms activation of the executioner phase of apoptosis, leading to DNA fragmentation and cellular dismantling. In the ML group, Caspase-3 expression (1.05 ± 0.10) remained comparable to controls ($p > 0.05$). In the DOX + ML group, Caspase-3 expression decreased significantly to 1.55 ± 0.14 compared to the DOX group ($p < 0.001$), demonstrating a 44.6% reduction. This finding indicates that montelukast effectively inhibits the executioner phase of apoptosis, protecting hepatocytes from programmed cell death.

4. Discussion

This study demonstrates the multi-mechanism protective effects of montelukast on doxorubicin-induced liver injury. Our findings demonstrate that montelukast exerts a hepatoprotective effect by reducing oxidative stress, enhancing the antioxidant defense system, modulating apoptotic pathways, and improving histopathological damage.

Doxorubicin treatment significantly increased oxidative stress in the liver. The increase in MDA levels suggests that doxorubicin induces lipid peroxidation. This finding is consistent with the knowledge that doxorubicin causes oxidative stress by generating reactive oxygen species (ROS) via the redox cycle¹⁵. The decrease in SOD, CAT, and GPx activities indicates suppression of the antioxidant defense system. The decrease in GSH levels supports the depletion of cellular antioxidant capacity by doxorubicin¹⁶.

Montelukast treatment significantly improved all these oxidative stress parameters. In particular, the significant decrease in MDA levels and the partial restoration of antioxidant enzyme activities in the DOX+ML group demonstrate the antioxidant properties of montelukast. These findings suggest that montelukast has antioxidant effects in addition to leukotriene receptor antagonism¹⁷. Our molecular findings have demonstrated that montelukast has a modulatory effect on apoptotic pathways. The increase in Bax and Caspase-3 expression and the decrease in Bcl-2 expression in the doxorubicin group indicate activation of the intrinsic apoptotic pathway. This finding is consistent with the knowledge that doxorubicin triggers apoptotic cell death via mitochondrial dysfunction¹⁸. Montelukast treatment decreased Bax and Caspase-3 expression while increasing Bcl-2 expression, demonstrating the anti-apoptotic properties of montelukast. This effect suggests that montelukast may inhibit cytochrome c release by stabilizing the mitochondrial membrane¹⁹. Recent studies suggest that montelukast may modulate apoptotic signals via the nuclear factor kappa B (NF- κ B) and p53 pathways²⁰. Histopathological findings have shown that montelukast is effective in preventing doxorubicin-induced structural damage. Pathological changes such as congestion, pyknotic nuclei, vacuolization, and sinusoidal dilation observed in the doxorubicin group were significantly reduced with montelukast treatment. This protective effect may be related to montelukast's ability to both reduce oxidative stress and prevent apoptotic cell death²¹. Although the molecular mechanisms

underlying the hepatoprotective effects of montelukast have not been fully elucidated, recent studies suggest several possibilities. In addition to cysteinyl leukotriene receptor 1 (CysLT1) antagonism, montelukast is thought to be effective through additional mechanisms, such as peroxisome proliferator-activated receptor gamma (PPAR- γ) activation and nuclear factor erythroid 2-related factor 2 (Nrf2) pathway activation^{22,23}.

Several limitations of this study should be acknowledged. First, serum liver function biomarkers (ALT, AST, ALP) were not evaluated, which would have provided additional clinical validation of liver injury. Second, the relatively small sample size (n=6 per group) may limit statistical power; however, the large effect sizes observed (Cohen's $d > 2.8$) confirm robust biological significance. Third, a single-dose doxorubicin regimen was employed, which does not fully reflect the chronic exposure patterns in clinical settings. Fourth, findings from this animal model may not directly translate to humans, and clinical trials are warranted. Fifth, mechanistic studies using specific pathway inhibitors were not performed. Finally, the potential interaction between montelukast and doxorubicin's antitumor efficacy was not evaluated.

Despite these limitations, our findings provide robust evidence for the hepatoprotective effects of montelukast and support its potential as a therapeutic agent against DOX-induced liver injury.

The findings of this study suggest that montelukast may be a potential adjuvant treatment option against doxorubicin-induced hepatotoxicity. Montelukast's well-established safety profile and widespread clinical use provide advantages for this potential application²⁴. However, further studies are needed to translate these findings to clinical use.

5. Conclusion

This study demonstrates the multi-mechanism protective effects of montelukast against doxorubicin-induced liver injury. Montelukast's effects in reducing oxidative stress, enhancing antioxidant defenses, modulating apoptotic pathways, and preserving histological integrity make it a potential therapeutic agent for chemotherapy-associated hepatotoxicity.

Author Contributions: Authors reviewed the results and approved the final version of the manuscript.

Funding: The authors received no financial support for the research, authorship, and/or publication of this article.

Institutional Review Board Statement: This study was approved by the Çanakkale Onsekiz Mart University Faculty of Medicine Local Animal Experimentation Ethics Committee (Ethics number: 2022-2200189432).

Conflict of Interest: The authors declare that they have no financial conflict of interest with regard to the content of this report.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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