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# Myrtenol alleviates hepatorenal toxicity caused by carbon tetrachloride: The role of oxidative stress and apoptosis

**Melika Vesal [1#], Elham Hakimizadeh [2#], Ayat Kaeidi [1, 3], Jalal Hassanshahi [1, 3*]**

[1] Physiology-Pharmacology Research Center, Research Institute of Basic Medical Sciences, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
[2] Immunology of Infectious Diseases Research Center, Research Institute of Basic Medical Sciences, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
[3] Department of Physiology and Pharmacology, School of Medicine. Rafsanjan University of Medical Sciences, Rafsanjan, Iran

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**ABSTRACT**

***Objective(s):*** This study aimed to investigate the protective effects of myrtenol at various doses against $CCl_4$-induced hepatorenal toxicity in mice, focusing on oxidative stress and apoptosis.

***Materials and Methods:*** Thirty-six male mice were randomly divided into six groups: control, myrtenol (50 mg/kg), $CCl_4$, and $CCl_4$ + myrtenol (5, 25, or 50 mg/kg). $CCl_4$ (1 ml/kg, intraperitoneally) was administered to induce hepatorenal injury, followed by daily oral gavage of vehicle or myrtenol (5, 25, or 50 mg/kg) for four weeks. Serum biochemical markers (alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen [BUN], and creatinine) and oxidative stress parameters (MDA, SOD, GPx, TAC) were measured. Apoptosis markers (Bax, Bcl-2, cleaved caspase-3) were assessed by Western blotting. Histopathological analysis of liver and kidney tissues was performed using H&E staining.

***Results:*** $CCl_4$ administration significantly increased serum alanine aminotransferase, aspartate aminotransferase, BUN, and creatinine levels, as well as tissue MDA content, while reducing SOD and GPx activities and TAC ($P<0.01$). Myrtenol, particularly at 50 mg/kg, markedly attenuated these biochemical and oxidative alterations. Myrtenol also decreased Bax expression and cleaved caspase-3 protein and increased Bcl-2 protein in hepatorenal tissues ($P<0.05$). Histopathological scoring revealed that myrtenol significantly alleviated $CCl_4$-induced liver and kidney damage ($P<0.05$).

***Conclusion:*** Myrtenol provides dose-dependent protection against $CCl_4$-induced hepatorenal injury, likely through anti-oxidant and anti-apoptotic mechanisms. These findings support myrtenol's therapeutic potential for managing oxidative organ injuries.

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## Introduction

Carbon tetrachloride ($CCl_4$) is a detergent and toxic liquid used as a model to induce liver and kidney injury in animals by causing oxidative stress (1, 2). It is easily absorbed through the respiratory, skin, and digestive systems and damages body tissues. Cytochrome P450 transforms it into free radicals, which cause lipid peroxidation in cells. Free radicals induce necrosis and apoptosis (2, 3). The molecular mechanisms underlying $CCl_4$-induced acute liver and kidney damage are well documented. Through the action of the cytochrome P-450 enzyme system, $CCl_4$ is metabolized into chlorine and trichloromethyl radicals (4). After lipid peroxidation begins in these organs, the trichloromethyl radical rapidly reacts with molecular oxygen, generating additional free radicals (5). A recent review by Vimala *et al.* comprehensively summarized the damaging effects of $CCl_4$ on liver and kidney tissues, emphasizing that $CCl_4$-derived free radicals cause lipid peroxidation, disrupt mitochondrial function, and ultimately lead to cell death through ferroptosis, necrosis, and apoptosis (6). Similarly, Fareed *et al.* provided a systematic review of the molecular mechanisms of $CCl_4$-induced hepatotoxicity, highlighting the roles of inflammatory cytokines such as TNF-α and NO, as well as the involvement of apoptotic pathways (7). Acute kidney injury is one of the global problems associated with excessive mortality, morbidity, and healthcare costs (8). Also, acute liver injury is a global issue with a high rate of morbidity and mortality, and researchers are continually discovering new treatment solutions for it (9). Numerous original studies have specifically investigated $CCl_4$-induced hepatic and renal injury. Hermenean *et al.* demonstrated $CCl_4$-induced acute nephrotoxicity in mouse kidney (1), while Hakimizadeh *et al.* characterized $CCl_4$-induced hepatorenal injury and its amelioration by calcium dobesilate (3) and troxerutin (5). Unsal *et al.* reviewed $CCl_4$ toxicity, free radicals, and the role of anti-oxidants (2). Reviews on mitochondrial quality control in kidney injury and repair (8), oxidative stress and mitochondrial dysfunction in

*Corresponding author: Jalal Hassanshahi. Khalije Fars Blvd., Pistachio Co. Street, Rafsanjan University of Medical Sciences, Rafsanjan, Iran. Tel/Fax: +98-34-31315003, Email: hasanshahij@gmail.com, hasanshahij@rums.ac.ir
# These authors contributed equally to this work

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chronic kidney disease (10), and mitochondrial mechanisms of apoptosis and necroptosis in liver diseases (11) further support the relevance of these pathways in both organs.

Oxidative stress is one of the most important mechanisms of liver and kidney injury. It occurs when reactive oxygen species (ROS) production increases and the anti-oxidant complex is disrupted (1). Free radicals, particularly ROS, induce apoptosis through several cellular and molecular pathways. Because both the kidney and liver are rich in mitochondria and metabolically active, they are especially vulnerable to oxidative damage (10). Mitochondrial ROS triggered mitochondrial outer membrane permeabilization, then suppressed the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) and up-regulated the pro-apoptotic protein B-cell lymphoma 2-associated X (Bax) in the liver (12) and kidney (11). However, anti-oxidant defense system enzymes, including catalase, superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase, prevent tissue damage (13).

Myrtenol is a bicyclic monoterpene alcohol and an active element obtained from the myrtle (Myrtus communis) (14). Importantly, a growing body of hepatic and renal literature exists on myrtenol. A review by Mrabti *et al.* highlighted myrtenol's antimicrobial, anti-inflammatory, anti-oxidant, and anti-apoptotic pharmacological properties (15). Amirteimoury showed that myrtenol protects against cisplatin-induced acute kidney injury in mice by suppressing oxidative stress (16). Rajizadeh *et al.* demonstrated that myrtenol significantly attenuated liver enzyme elevation, pro-inflammatory cytokines, and oxidant levels in both intrahepatic and extrahepatic cholestasis models, while restoring hepatic anti-oxidant defenses (17). These findings collectively support myrtenol's potential to mitigate oxidative organ injury, including in the liver and kidney.

However, despite these hepatic and renal findings, the effect of myrtenol on combined $CCl_4$-induced hepatorenal toxicity remains poorly defined. Moreover, most previous studies used a single dose or focused on only one organ, and no study has systematically compared multiple doses of myrtenol in a $CCl_4$-induced hepatorenal injury model. Therefore, the novelty of the present study is threefold: It evaluates myrtenol in a combined liver-kidney toxicity model; It examines a dose-response range of 5, 25, and 50 mg/kg; and it integrates serum functional markers, tissue oxidative stress parameters, apoptotic protein expression, and histopathological damage scores in both organs. This design may provide a more comprehensive, translational basis for using myrtenol in oxidative organ injuries. Therefore, this study aims to evaluate the protective effects of myrtenol at various dosages on carbon tetrachloride-induced hepatic and renal toxicity in mice, potentially providing a complementary therapeutic approach to mitigate these injuries.

## Materials and Methods

### *Animal*

This investigation was conducted as an *in vivo* experimental study. A total of 36 male BALB/c mice, each weighing approximately 30 ± 3 grams, were procured from the animal facility at Rafsanjan University of Medical Sciences. Before the experiment, all animals were housed under standardized conditions, including 45% relative humidity, an ambient temperature of 20-23°C, and unrestricted access to food and water for a one-week acclimation period. The study procedures adhered to the Animal Care and Use Guidelines outlined by the National Institutes of Health (NIH Publication No. 85-23, revised 2010). Ethical approval for the study was granted by the Rafsanjan University of Medical Sciences Ethics Committee (approval code: IR.RUMS.REC.1401.243).

### *Experimental groups*

The mice were randomly assigned to six groups, each consisting of six animals, as detailed below:

Group 1 (Control): Mice received an intraperitoneal (IP) injection of olive oil, serving as the vehicle for $CCl_4$, on day 1. One hour post-injection, they received saline via oral gavage once daily for four weeks (5, 18).

Group 2 (Myrtenol): This group was given an IP injection of olive oil on day 1. One hour later, myrtenol was administered orally at 50 mg/kg daily for four weeks.

Group 3 ($CCl_4$): Mice were injected intraperitoneally with $CCl_4$ (1 ml/kg) diluted 1:1 in 50% olive oil on day 1. Subsequently, they received daily oral saline gavage for four weeks, starting one hour after injection.

Group 4 ($CCl_4$ + Myrtenol 5 mg/kg): Animals were administered $CCl_4$ (1 ml/kg in 50% olive oil) via IP injection on day 1, followed by daily oral gavage of myrtenol at 5 mg/kg beginning one hour post-injection and continuing for four weeks.

Group 5 ($CCl_4$ + Myrtenol 25 mg/kg): This group received the same $CCl_4$ IP injection as above on day 1, followed by daily oral myrtenol at 25 mg/kg for four weeks, starting one hour after $CCl_4$ exposure.

Group 6 ($CCl_4$ + Myrtenol 50 mg/kg): Mice were injected with $CCl_4$ (1 ml/kg in 50% olive oil) intraperitoneally on the first day. One hour later, they were treated with myrtenol at 50 mg/kg via oral gavage daily for four weeks.

### *Study procedure*

In the present study, $CCl_4$ (1 ml/kg) was dissolved in 50% olive oil and injected intraperitoneally into the animals (1). On day 1, animals received either olive oil or $CCl_4$ (1 ml/kg) by injection. One hour post-injection, select groups received daily oral gavage of myrtenol at doses of 5, 25, or 50 mg/kg (19). Twenty-four hours following the final treatment, mice were anesthetized intraperitoneally with ketamine (100 mg/kg) and xylazine (10 mg/kg) (Sigma, St. Louis, USA). The depth of anesthesia was confirmed using a paw pinch reflex before blood collection. Blood samples were obtained via the orbital sinus and subsequently centrifuged at 3000 × g for 20 min. Serum was separated and stored in sterile tubes at −80 °C until further analysis.

Following blood collection, animals were deeply anesthetized, and kidney and liver tissues were excised and immediately divided into two portions. One portion was fixed in 10% formalin for histopathological evaluation using hematoxylin and eosin (H&E) staining. The other portion was homogenized in ice-cold buffer, centrifuged at 6000 × g for 20 min, and the resulting supernatant was collected and stored at −80 °C for subsequent biochemical assays, including western blot analysis of apoptosis-related proteins and assessment of oxidative stress markers using appropriate commercial kits.

### *Biochemical parameter measurement*

Following blood collection, serum was separated for

**Table 1.** Effect of myrtenol on mouse kidney and liver functional indicators

| Group | BUN (mg/dl) | Cr (mg/dl) | ALT (U/l) | AST (U/l) |
|---|---|---|---|---|
| Control | 21.3±4.60 | 0.51±0.11 | 264±12.6 | 71.1±9.41 |
| Myrtenol 50 mg/kg | 20.1±3.04 | 0.54±0.07 | 263±13.9 | 70.1±8.01 |
| $CCl_4$ | 34.3±4.10*** | 0.72±0.11** | 299±13.8** | 95.3±5.08*** |
| $CCl_4$ + Myrtenol 5 mg/kg | 34.0±1.60 | 0.70±0.09 | 289±12.3 | 94.6±9.52 |
| $CCl_4$ + Myrtenol 25 mg/kg | 26.8±6.00 | 0.71±0.10 | 290 ±10.3 | 93.6±6.71 |
| $CCl_4$ + Myrtenol 50 mg/kg | 25.1±5.40# | 0.54±0.06# | 258±19.9## | 80.1±4.07# |

Values are presented as mean±SD. **$P$<0.01 and ***$P$<0.001 compared with the control. #$P$<0.05 and ##$P$<0.01 compared to the $CCl_4$
BUN: Blood Urea Nitrogen; Cr: Creatinine; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase

analysis. Subsequently, serum concentrations of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were quantified using commercial assay kits (Pars Azmoon Co., Tehran, Iran) to assess hepatic function. Additionally, blood urea nitrogen (BUN) and serum creatinine (Cr) levels were measured employing standardized commercial kits (Pars Azmoon Co., Tehran, Iran) to evaluate renal function (5).

### *Oxidative stress parameters*

To assess oxidative stress, supernatants from kidney and liver tissues preserved in liquid nitrogen were analyzed. The enzymatic activities of GPx and SOD, as well as total antioxidant capacity (TAC) and malondialdehyde (MDA) levels, were quantified in the kidney and liver tissue samples using commercial assay kits (ZellBio, Germany). All assays were performed under the manufacturer's protocols utilizing an automated microplate reader (BioTek, USA) (3).

### *Apoptosis parameters*

Western blot was used to assess apoptosis-related proteins (Bcl-2, Bax, and cleaved caspase-3) in liver and kidney tissues. Proteins (50 µg) were separated by 12.5% SDS-PAGE and transferred to nitrocellulose membranes. After overnight blocking at 4 °C with 5% non-fat milk in TBST (pH 7.4), membranes were incubated for 3 h at room temperature with primary antibodies against Bcl-2 (CST #3498; 1:1000), Bax (Abcam ab182733; 1:1000), and caspase-3 (CST #9664; 1:1000) in blocking buffer. Following three TBST washes, membranes were incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit secondary antibody (Abcam ab205718; 1:5000). Bands were visualized by ECL and quantified with ImageJ, using β-actin (Abcam ab115777; 1:5000) as the loading control (20, 21).

### *Histopathological analysis*

Liver and kidney sections were stained with H&E and examined under a light microscope (Nikon Labophot, Japan) at 100×. Histopathological damage scores (LTDS and KTDS) were assessed blindly in six sections per mouse. Kidney sections were semi-quantitatively scored for glomerular atrophy, inflammatory cell infiltration, tubular necrosis, and tubulointerstitial damage according to established criteria (3, 5). Liver sections were evaluated for pyknosis and congestion. Lesions were graded as mild (<25% involvement), moderate (<50%), marked (<75%), or severe (>75%). Total damage was scored 0–3: 0–0.5, normal; 0.51–0.99, minor; 1–1.99, moderate; and >2, severe. All image evaluations and scoring were repeated independently by multiple observers.

### *Data analysis*

Data analysis was conducted using SPSS Statistics version 22 and GraphPad Prism software (version 6.01, GraphPad Software, USA). For parametric variables, group comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Nonparametric data were analyzed using the Kruskal-Wallis test. Results are expressed as mean±standard deviation (SD), with statistical significance defined as $P$<0.05.

## Results

### *The effect of myrtenol on biochemical parameters*

Our results showed that serum Cr ($P$<0.01) and BUN ($P$<0.001) levels were higher in mice receiving $CCl_4$ than in the control group (Table 1). Likewise, serum AST ($P$<0.001) and ALT ($P$<0.01) levels were higher in animals receiving $CCl_4$ than in the control group (Table 1).

Myrtenol with a dose of 50 mg/kg decreased serum Cr ($P$<0.05) and BUN ($P$<0.05) in mice treated by myrtenol ($CCl_4$+Myrtenol 50 mg/kg group) versus the group that received $CCl_4$ (Table 1), likewise AST ($P$<0.05) and ALT ($P$<0.01) in serum were reduced in the $CCl_4$+Myrtenol 50 mg/kg group compared to the group received $CCl_4$ (Table 1).

### *Effect of myrtenol on oxidative stress parameters*

MDA levels in kidney ($P$<0.01) and liver ($P$<0.001) tissues were significantly elevated in animals that received $CCl_4$ compared with the control group (Figures 1A and 2A). In addition, the MDA levels in the kidney ($P$<0.05) and liver ($P$<0.01) were significantly decreased in mice treated with myrtenol at doses of 25 and 50 mg/kg ($CCl_4$+Myrtenol 25 mg/kg and $CCl_4$+Myrtenol 50 mg/kg groups) compared to the group that received $CCl_4$ alone (Figures 1A and 2A).

The results of the present study showed that the activity of the SOD enzyme, a key component of the body's antioxidant defense system, was significantly reduced in the kidney ($P$<0.01) and liver ($P$<0.001) tissues of animals receiving $CCl_4$ compared to the control group (Figures 1B and 2B). However, myrtenol at 50 mg/kg ($CCl_4$+Myrtenol 50 mg/kg group) significantly increased SOD activity in kidney tissue compared with the $CCl_4$ group (Figure 1B; $P$<0.05). Additionally, treatment with myrtenol at doses of 25 mg/kg and 50 mg/kg ($CCl_4$+Myrtenol 25 mg/kg and $CCl_4$+Myrtenol 50 mg/kg groups) resulted in increased GPx enzyme activity in the liver tissue of mice compared to those that received only $CCl_4$ ($P$<0.05 and $P$<0.01, respectively; Figure 2B).

GPx activity in kidney ($P$<0.001) and liver ($P$<0.001) tissues was significantly reduced in animals that received

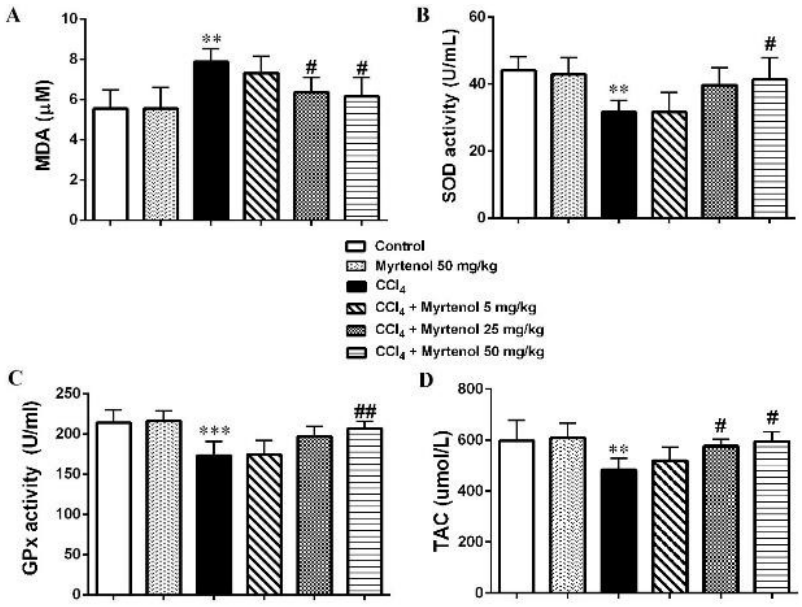



**Figure 1.** Effect of myrtenol on mouse kidney oxidative stress parameters
Values are presented as mean±SD. **$P$<0.01 and ***$P$<0.001 compared with the control. #$P$<0.05 and ##$P$<0.01 compared to the $CCl_4$

$CCl_4$ compared with the control group (Figures 1C and 2C). However, myrtenol at 50 mg/kg ($CCl_4$+Myrtenol 50 mg/kg group) significantly increased GPx activity in kidney tissue compared with the $CCl_4$ group (Figure 1C; $P$<0.01). Additionally, GPx activity in the liver tissue was significantly enhanced in animals treated with myrtenol at doses of 25 mg/kg and 50 mg/kg ($CCl_4$+Myrtenol 25 mg/kg and $CCl_4$+Myrtenol 50 mg/kg groups) compared to the $CCl_4$ group ($P$<0.05 and $P$<0.01, respectively; Figure 2C).

The results also showed that TAC levels were significantly reduced in kidney and liver tissues of animals receiving $CCl_4$ compared with the control group ($P$<0.01). Additionally, treatment with myrtenol at doses of 25 mg/kg and 50 mg/kg ($CCl_4$+Myrtenol 25 mg/kg and $CCl_4$+Myrtenol 50 mg/kg groups) resulted in increased TAC levels in the kidney tissue of mice compared to those that received only $CCl_4$ ($P$<0.05; Figure 1D). Furthermore, myrtenol at 50 mg/kg ($CCl_4$+Myrtenol 50 mg/kg group) significantly increased TAC levels in liver tissue compared with $CCl_4$ alone ($P$<0.05; Figure 2D).

### *Effect of myrtenol on hepatorenal tissue apoptosis*

Our findings showed that $CCl_4$ administration significantly increased Bax protein expression in mouse kidney and liver tissues compared with the control ($P$<0.01; Figures 3A and 4A). Furthermore, myrtenol at 50 mg/kg markedly reduced Bax protein expression in both kidney ($P$<0.05) and liver ($P$<0.01) tissues of $CCl_4$-administered mice ($CCl_4$+Myrtenol 50 mg/kg group) relative to the $CCl_4$ group (Figures 3A and 4A).

Based on our findings, $CCl_4$ significantly reduced Bcl-2 protein expression in kidney and liver tissues compared

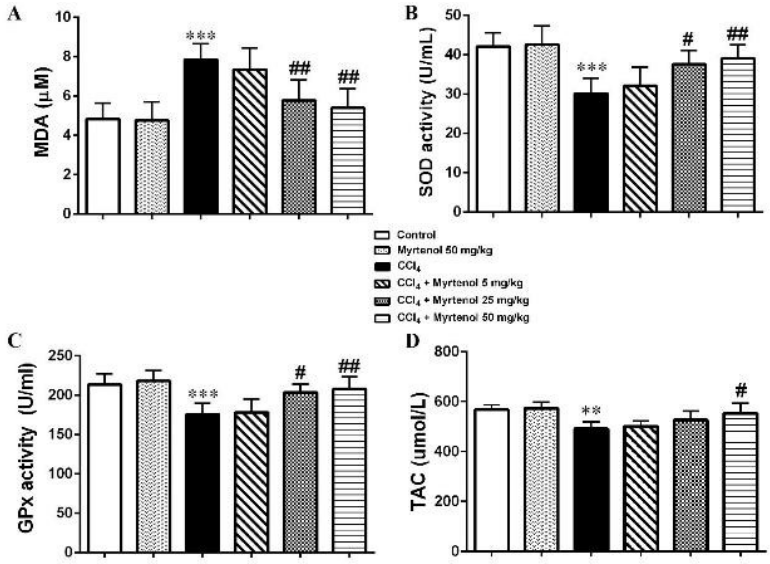



**Figure 2.** Effect of myrtenol on mouse liver oxidative stress parameters
Values are presented as mean±SD. **$P$<0.01 and ***$P$<0.001 compared with the control. #$P$<0.05 and ##$P$<0.01 compared to the $CCl_4$

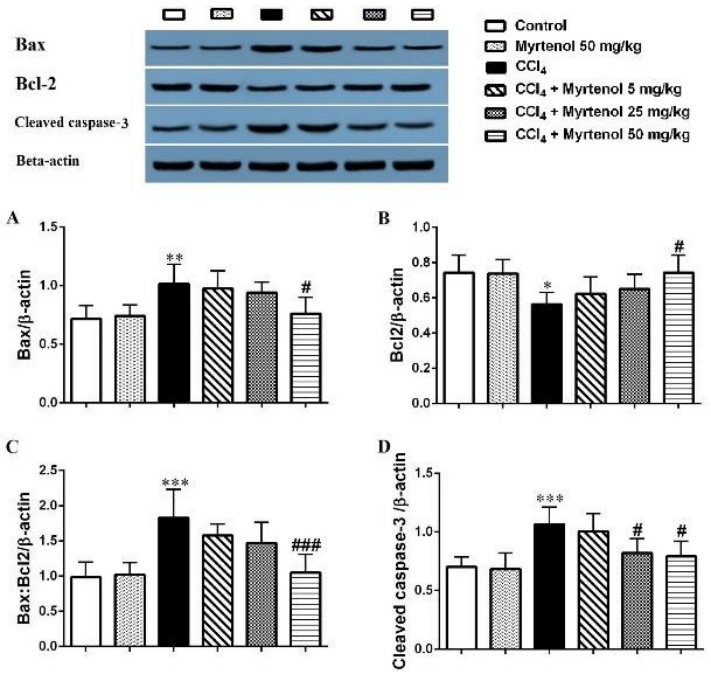



**Figure 3.** Effect of myrtenol on mouse kidney apoptosis parameters (Bax (A), Bcl-2 (B), Bax: Bcl-2 ratio (C), and cleaved caspase-3 (D) protein expression) Values are presented as mean±SD. **P*<0.05, **P<0.01, and ****P*<0.001 compared to the control. #*P*<0.05, and ###*P*<0.001 compared to the $CCl_4$

with the control (*P*<0.05; Figures 3B and 4B). Additionally, myrtenol at 50 mg/kg significantly increased Bcl-2 expression in kidney and liver tissues of $CCl_4$-treated mice ($CCl_4$+Myrtenol 50 mg/kg group) compared with the $CCl_4$-alone group (*P*<0.05; Figures 3B and 4B).

Our results also showed that $CCl_4$ increased cleaved caspase-3 protein expression in the hepatorenal tissues of mice treated with $CCl_4$ compared with the control (*P*<0.001; Figures 3D and 4D). Moreover, cleaved caspase-3 protein expression decreased significantly in the kidney (*P*<0.05) and liver tissues (*P*<0.05 and *P*<0.01, respectively) of mice treated with myrtenol at doses of 25 mg/kg and 50 mg/kg ($CCl_4$ + Myrtenol 25 mg/kg and $CCl_4$+Myrtenol 50 mg/kg groups) compared with the group that received $CCl_4$ alone (Figures 3D and 4D).

Furthermore, myrtenol at 50 mg/kg decreased the Bax:Bcl-2 ratio in kidney tissue in the $CCl_4$+Myrtenol 50 mg/kg group compared with the $CCl_4$ group (*P*<0.001; Figure 3C). Moreover, myrtenol significantly lowered the Bax:Bcl-2 ratio in the liver tissue of mice treated with 25 mg/kg and 50 mg/kg doses ($CCl_4$ + Myrtenol 25 mg/kg and $CCl_4$ + Myrtenol 50 mg/kg groups) compared to the $CCl_4$

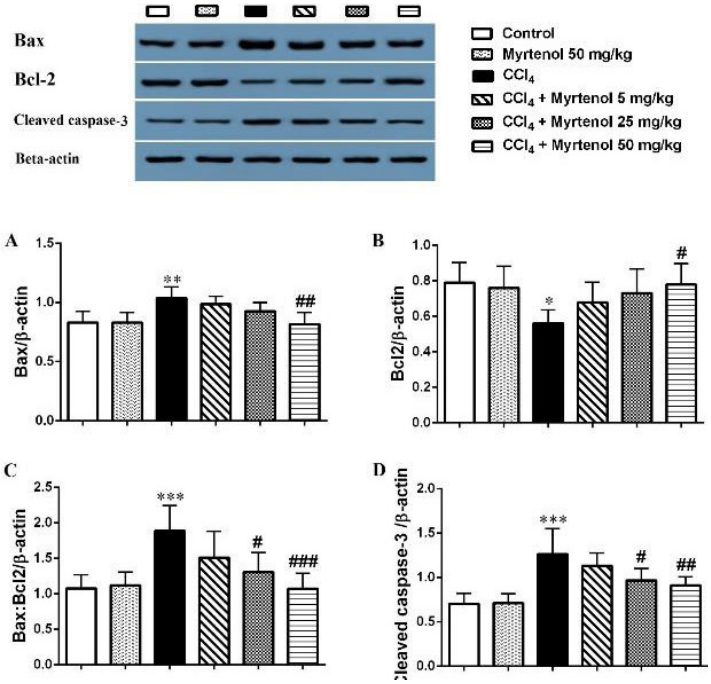



**Figure 4.** Effect of myrtenol on mouse liver apoptosis parameters (Bax (A), Bcl-2 (B), Bax: Bcl-2 ratio (C), and cleaved caspase-3 (D) protein expression) Values are presented as mean±SD. **P*<0.05, **P<0.01, and ****P*<0.001 compared to the control. #*P*<0.05, ##*P*<0.01, and ###*P*<0.001 compared to the $CCl_4$

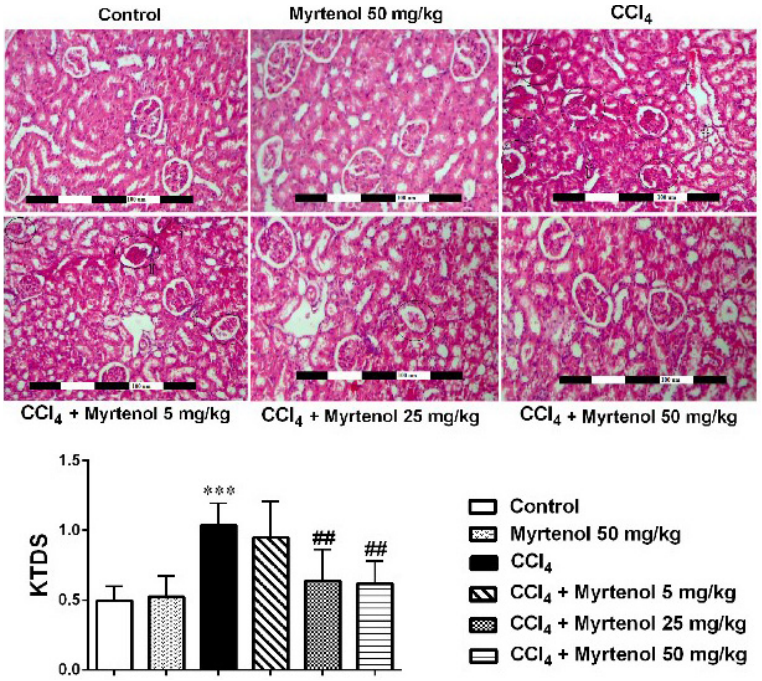



**Figure 5.** Mouse kidney hematoxylin and Eosin-stained sections (100× magnification) in the experimental groups
Scores 1–3 were assigned to leukocyte infiltration, glomerular atrophy, and tubular necrosis in the kidney tissue sections. However, scores 0 to 0.5 were considered normal tissue. Arrow: leukocyte infiltration. Circle: tubular necrosis/glomerular atrophy. KTDS: Kidney tissue damage score. Values are presented as mean±SD. ***$P$<0.001 compared to the control. ##$P$<0.01 compared to the $CCl_4$

group ($P$<0.05 and $P$<0.001, respectively; Figure 4C).

***Effect of myrtenol on hepatorenal tissue injury***

Our H&E staining results showed that the control group exhibited no pathological changes in liver and kidney tissues (Figures 5 and 6). However, $CCl_4$ significantly increased both LTDS and KTDS in $CCl_4$-treated mice compared with the control ($P$<0.001; Figures 5 and 6). Additionally, myrtenol at doses of 25 and 50 mg/kg mitigated the injury induced by $CCl_4$, as evidenced by a significant reduction in KTDS ($P$<0.01; Figure 5) and LTDS ($P$<0.05 and $P$<0.01, respectively; Figure 6) in the $CCl_4$+Myrtenol 25 mg/kg and

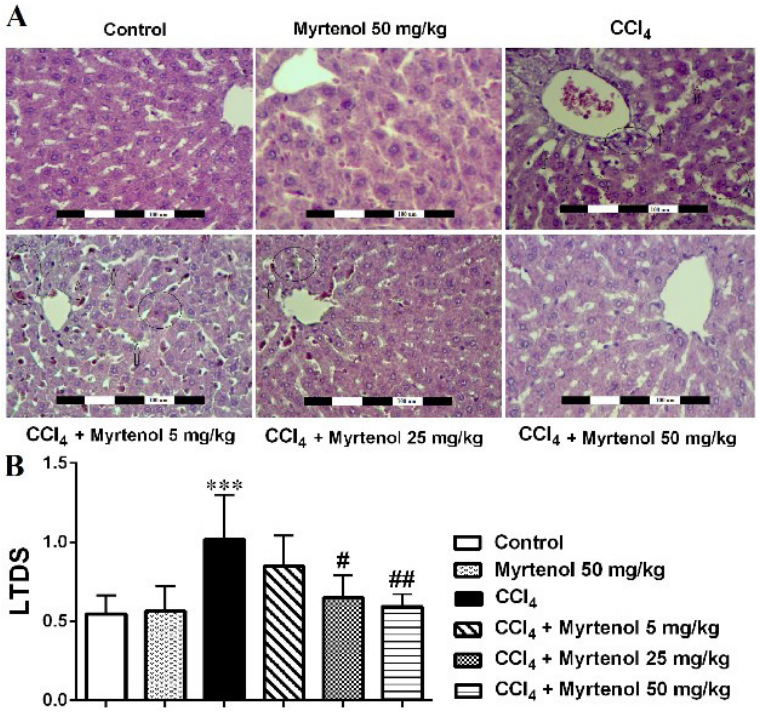



**Figure 6.** Mouse liver hematoxylin and eosin-stained sections (100× magnification) in the experimental groups
Scores 1–3 were assigned to congestion and pyknotic cells in liver tissue sections. However, scores 0 to 0.5 were considered normal tissue. Circle: Congestion; Arrow: Fat deposition/pyknosis. LTDS: Liver tissue damage score. Values are presented as mean±SD. ***$P$<0.001 compared to the control. #$P$<0.05 and ##$P$<0.01 compared to the $CCl_4$

$CCl_4$+Myrtenol 50 mg/kg groups compared to the group that received $CCl_4$ alone.

## Discussion

Because hepatorenal injuries are clinically significant, extensive research has evaluated the effectiveness of different substances in controlling these injuries through anti-inflammatory, anti-oxidant, and anti-apoptotic mechanisms (3, 5, 22). Moreover, some previous studies have investigated the diverse therapeutic effects of herbal medicines, such as myrtenol, in mitigating oxidative stress, inflammation, and apoptosis (23, 24). In this regard, several studies have confirmed myrtenol's protective effects on the brain (14), heart (25), stomach (19), and lungs (26) against cytotoxic agents by activating the anti-oxidant system. Additionally, Selvaraj *et al.* demonstrated that myrtenol had antibacterial effects against methicillin-resistant Staphylococcus aureus due to its anti-oxidant properties and its ability to inhibit the ROS system (27).

This research showed that $CCl_4$ administration significantly increased levels of various functional biomarkers, including ALT, AST, BUN, and Cr, indicating hepatic and renal injury. These findings align with Unsal *et al.* They reported that $CCl_4$ induced oxidative stress by increasing ROS production, which ultimately damaged multiple organs, such as the liver and kidneys. It led to elevated BUN, Cr, AST, and ALT levels in the blood (2). Our findings also indicated that myrtenol at 50 mg/kg could ameliorate hepatorenal injuries by decreasing these parameters. In this context, Amirteimoury *et al.* suggested that the same doses of myrtenol could significantly reduce serum BUN and Cr levels in a rat model of cisplatin-induced kidney injury (16). Similarly, another study found that a component of Myrtus communis could lower ALT and AST in rats administered $CCl_4$ (28).

More recent evidence has further expanded myrtenol's pharmacological relevance. Bejeshk *et al.* demonstrated that myrtenol-loaded niosomes can prevent lung ischemia-reperfusion injury in rats by balancing the NF-κB signaling pathway (29). This is particularly relevant to $CCl_4$-induced hepatorenal toxicity because $CCl_4$-derived trichloromethyl radicals initiate lipid peroxidation and inflammatory signaling (2). Although we did not directly measure NF-κB, the anti-oxidant and anti-apoptotic effects observed in the present study are probably consistent with myrtenol-mediated modulation of these pathways (30).

Our results also showed increased MDA activity with a simultaneous reduction in SOD, GPx, and TAC levels in renal and hepatic tissues of mice exposed to a toxic dose of $CCl_4$. In contrast, myrtenol (50 mg/kg) increased SOD, GPx, and TAC activities while reducing MDA levels in treated mice. Research by Amin and colleagues also showed that myrtenol could inhibit the oxidant system by elevating GPx, SOD, and TAC activity in liver tissue and reducing MDA concentration in a paraquat-induced hepatotoxicity model (31). Similarly, some studies found that myrtenol and myrtenal ameliorate oxidative stress by lowering MDA activity and elevating SOD activity in kidney and liver tissues (32, 33).

Moreover, Rajizadeh *et al.* showed that myrtenol increased GPx and SOD activity in the serum and lungs of rats with asthma (24). Xuemei *et al.* also investigated myrtenol's ameliorative effects against gestational diabetes in rats. They showed that myrtenol protected rats against gestational diabetes by reducing MDA content and increasing anti-oxidant biomarkers (34). In addition, Viana *et al.* indicated that myrtenol mitigates ethanol-induced damage by reducing MDA concentration and inhibiting the ROS system (19).

The apoptotic regulatory proteins play a consequential role in hepatorenal injury (5). Activation of the oxidant system through membrane lipid peroxidation and DNA damage causes renal injury, ultimately leading to apoptosis and cell death (35). DNA damage can influence the expression and activation of proteins, such as Bcl-2 and caspase-3, tipping the balance towards apoptosis (36). In our study, $CCl_4$ triggered the apoptosis pathway by increasing Bax expression, cleaved caspase-3, and the Bax:Bcl-2 ratio in kidney and liver tissues (Figures 3 and 4). In our study, myrtenol at 50 mg/kg decreased Bax, cleaved caspase-3, and the Bax:Bcl-2 ratio while increasing Bcl-2 protein levels in hepatorenal tissues. Myrtenol has also been shown to protect against cerebral and cardiac ischemic injury by increasing Bax and caspase-3 levels while inactivating Bcl-2 (14, 25). Consistent with our study, myrtenol was shown to exert an anti-apoptotic effect by down-regulating Bax and caspase-3 and up-regulating Bcl-2 in treated skin tissues, increasing skin flap survival (37). Because the liver and kidney are mitochondria-rich organs and mitochondrial ROS contribute to $CCl_4$-induced apoptosis (10-12), preserving mitochondrial quality control may contribute to the hepatorenal protection seen in our study; however, this remains to be directly tested. Myrtenol has also been shown to promote skin flap survival by inhibiting apoptosis (38), which aligns with our finding that myrtenol decreased Bax and cleaved caspase-3 and increased Bcl-2 in both liver and kidney. A recent comprehensive review also highlighted myrtenol as a promising terpene with potent anti-oxidant, anti-inflammatory, and anti-apoptotic pharmacological properties (39). Our dose-response data extend these observations to a combined $CCl_4$-induced hepatorenal toxicity model and provide parallel functional, oxidative, apoptotic, and histopathological evidence in both organs.

Our study also revealed that $CCl_4$ administration significantly elevated liver and kidney tissue injury. Moreover, treatment with myrtenol at 25 and 50 mg/kg effectively attenuated these histopathological injuries, demonstrating a dose-dependent protective effect against $CCl_4$-induced hepatorenal damage. Consistent with our findings, Amirteimoury *et al.* (16) showed that myrtenol at 25 and 50 mg/kg significantly attenuated cisplatin-induced nephrotoxicity by reducing histopathological damage and oxidative stress in rat kidney tissue. Similarly, Amin *et al.* (31) reported that myrtenol mitigated paraquat-induced hepatotoxicity, improving liver histology and enhancing anti-oxidant enzyme activities. These studies further support myrtenol's dose-dependent protective role against chemically induced hepatorenal injuries.

Compared with previous myrtenol studies that often used a single dose or focused on only one organ (14, 16, 19, 25, 26, 29-31, 38), the present study provides a more integrated comparison by evaluating three doses (5, 25, and 50 mg/kg) in a combined $CCl_4$-induced hepatorenal toxicity model. This is relevant because $CCl_4$ simultaneously affects the liver and kidney through shared cytochrome P450-dependent bioactivation and oxidative stress mechanisms (1, 3, 5). The parallel assessment of serum functional markers, tissue oxidative stress parameters, apoptotic

protein expression, and histopathological damage scores in both organs strengthens the translational relevance of these findings. It supports myrtenol's potential as a multi-organ protective agent. Nevertheless, further pharmacokinetic, chronic toxicity, and clinical studies are needed to confirm myrtenol's translational potential.

Despite previous reports on the anti-oxidant and anti-apoptotic properties of myrtenol, the present study is novel because it is the first, to our knowledge, to systematically evaluate dose-dependent effects of myrtenol in a $CCl_4$-induced combined hepatorenal toxicity model. The parallel assessment of liver and kidney function, oxidative stress, apoptosis, and histopathology provides a comprehensive preclinical rationale for further investigation. However, further pharmacokinetic, chronic toxicity, and clinical studies are needed to confirm myrtenol's translational potential.

This study has some limitations. Only male mice were used, and the protective effects of myrtenol were evaluated at a single time point after $CCl_4$ administration. In addition, direct measurement of reactive oxygen species and mitochondrial function was not performed. Future studies should examine female mice, multiple time points, pharmacokinetic profiles, and mitochondrial apoptotic pathways to clarify myrtenol's translational potential further. Also, although baseline ALT was higher than typical reference ranges, all groups were handled identically (Same kit).

## Conclusion

Our study showed that myrtenol dose-dependently protects against $CCl_4$-induced hepatorenal injury in mice, mainly through anti-oxidant and anti-apoptotic effects. At 50 mg/kg, myrtenol significantly reduced serum ALT, AST, BUN, and creatinine, decreased MDA, and increased SOD, GPx, and TAC. It also attenuated hepatorenal apoptosis by down-regulating Bax and cleaved caspase-3 and lowering the Bax:Bcl-2 ratio. Histopathology confirmed reduced tissue damage in treated groups. These findings highlight myrtenol's therapeutic potential for oxidative stress-mediated organ injury and support further clinical investigation.

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## Availability of Data and Materials

Data can be obtained upon request from the corresponding author.

## Ethical Approval and Consent to Participation

The Ethics Committee of Rafsanjan University of Medical Sciences reviewed and approved all experimental protocols (Ethics No. IR.RUMS.REC.1401.243), and all procedures were conducted under the guidelines for the care and use of laboratory animals as outlined in the National Institutes of Health Publication No. 85-23, revised 2010.

## Authors' Contributions

J H conceived and designed the experiments; E H, A K, M V, and J H performed the experiments; A K and J H analyzed the data; A K and J H contributed reagents, materials, and analysis tools; and E H, M V, and J H wrote the article. All authors agree to be accountable for all aspects of the work, ensuring integrity and accuracy. The authors declare that all data were generated in-house and that no paper mill was used.

## Conflicts of Interest

The authors declare no conflicts of interest.

## Declaration

We have not used any AI tools or technologies to prepare this manuscript.

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