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Protective Effects of Morin Against Paracetamol-Induced Nephrotoxicity via Modulation of Oxidative Stress, Inflammation, and Apoptosis Pathways

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Abstract: Paracetamol (PCM), although a widely used and non-prescription effective analgesic and antipyretic, can cause nephrotoxicity by causing kidney damage at high doses. Morin (MRN) is a flavonoid found in white mulberry and cranberry, with low toxicity and prominent antioxidant, anti-inflammatory, and antiapoptotic properties. This study aimed to investigate the effects of MRN on PCM-induced kidney toxicity by biochemical, molecular, and histopathological methods.Thirty-five Wistar rats were divided into Control, MRN (100 mg/kg), PCM (1000 mg/kg), PCM+MRN50 (50 mg/kg), and PCM+MRN100 (100 mg/kg) groups (n=7); the substances were administered orally for 6 days. The blood and kidney tissues were evaluated with biochemical (urea, creatinine, MDA, GSH, SOD, CAT, GPx), genetic (NF-κB, TNF-α, Caspase-3, Bax, Bcl-2, KIM-1, AQP2) and histopathological methods. While PCM negatively affected kidney function tests, oxidative stress levels, inflammation, and apoptosis markers and caused kidney damage, MRN suppressed these negative effects at both doses, improved kidney function, antioxidant defense, inflammation, and apoptosis parameters, and reduced histopathological damage. The study found that MRN effectively protected kidney tissue from PCM-induced damage by reversing oxidative stress, inflammation, and apoptosis.

Morin'in Oksidatif Stres, İnflamasyon ve Apoptozis Yollarının Modülasyonu Yoluyla Parasetamol Kaynaklı Nefrotoksisiteye Karşı Koruyucu Etkileri

Anahtar Kelimeler
Apoptozis, İnflamasyon, Morin, Nefrotoksisite, Oksidatif stres, Parasetamol

Öz: Paracetamol (PCM), yaygın ve reçetesiz kullanılan etkili bir analjezik ve antipiretik olsa da, yüksek dozlarda böbrek hasarına neden olarak nefrotoksisiteye yol açabilir. Morin (MRN), beyaz dut ve kızılcıkta bulunan, düşük toksisiteye sahip, antioksidan, antiinflamatuvar ve antiapoptotik özellikleriyle öne çıkan bir flavonoiddir. Bu çalışmada, PCM'nin neden olduğu böbrek toksisitesi üzerine MRN'nin etkileri biyokimyasal, moleküler ve histopatolojik yöntemlerle araştırılması amaçlanmıştır. Otuz beş Wistar sıçan, Kontrol, MRN (100 mg/kg), PCM (1000mg/kg), PCM+MRN50 (50mg/kg) ve PCM+MRN100 (100mg/kg) gruplarına ayrılmış (n=7); maddeler 6 gün boyunca oral uygulanmıştır. Alınan kan ve böbrek dokuları biyokimyasal (üre, kreatinin, MDA, GSH, SOD, KAT, GPx), genetik (NF-κB, TNF-α, Caspase-3, Bax, Bcl-2, KIM-1, AQP2) ve histopatolojik yöntemlerle değerlendirilmiştir. PCM böbrek fonksiyon testlerini, oksidatif stres düzeylerini, inflamasyon ve apoptoz belirteçlerini olumsuz etkileyip böbrek hasarı oluştururken; MRN her iki dozda bu olumsuz etkileri baskılayarak böbrek fonksiyonu, antioksidan savunma, inflamasyon ve apoptoz parametrelerini iyileştirmiş, histopatolojik hasarı azaltmıştır. Çalışmada MRN'nin oksidatif stresi, inflamasyonu ve apoptozu tersine çevirerek böbrek dokusunu PCM kaynaklı hasardan etkili bir şekilde koruduğu bulunmuştur.

1. INTRODUCTION

The deterioration of the kidney's main function, detoxification and elimination, is defined as nephrotoxicity due to exogenous and endogenous toxic substances. Drugs used in clinical settings can damage the kidneys due to their role in elimination and can be the cause of drug-induced nephrotoxicity [1]. Although paracetamol (PCM) is an easily available, effective, and convenient drug that is sold without a prescription in most countries, it has serious side effects at high doses. PCM, which is widely used as a pain reliever and antipyretic, causes liver and kidney failure at excessive doses [2]. The use of 200 mg/kg and above is the main cause of liver toxicity and also causes nephrotoxicity. Excessive PCM use causes liver necrosis in 10-40% of patients, while kidney failure is observed in 2% [3]. Once PCM is taken, a small fraction—around 2–4%—is metabolized in the liver by cytochrome P450 enzymes into a highly electrophilic and toxic compound known as N-acetyl-p-benzoquinone imine (NAPQI). Normally, this metabolite is rendered harmless by conjugation with glutathione and is eliminated through the urine. However, if NAPQI levels exceed the available glutathione stores, the excess compound builds up and can result in damage to both liver and kidney tissues [4]. Morin (MRN), which is commonly found in white mulberry and cornelian cherry, is a flavonoid with antioxidant, anti-inflammatory, antiautophagic, and antiapoptotic properties [5]. The most important feature of MRN is that it reduces oxidative stress by inhibiting ROS production [6]. MRN shows a tissue protective effect against oxidative stress by regulating cytochrome P450 activity, inhibiting ROS production, increasing antioxidant genes and Bcl-2 expression, and reducing apoptotic factor release, even at high concentrations without toxic effects [7]. Additionally, MRN exhibits anti-inflammatory effects by inhibiting the Nuclear factor kappa B (NF-κB) signaling pathway [8]. Also, having minimum toxicity even at high doses provides an important advantage to MRN [9]. It has been reported by Çomaklı et al. [10] that MRN has a protective effect against oxidative stress-induced damage in glomerular mesenchymal cells in the kidney.

The presented study aimed to investigate the effects of MRN on PCM-induced kidney toxicity, which is widely used as an analgesic and antipyretic, by biochemical, molecular, and histopathological methods.

2. MATERIAL AND METHOD

2.1. Chemicals

PCM (Parol, 500 mg/tablet) was obtained from Atabay Chemical Company (Istanbul, Türkiye), and MRN (Cas No: 654055-01-3) was obtained from Sigma Chemical Company (USA).

2.2. Experimental Animals

The study involved 35 Wistar albino rats, each weighing between 220–250 grams and aged 10 to 12 weeks. The animals were housed in cages within a temperature-controlled environment (24–25 °C) under a 12-hour light/dark cycle. They had free access to standard feed and water throughout the study. All experimental procedures were conducted at the KONUDAM Experimental Medicine Practice and Research Center.

2.3. Ethics Committee Approval

Ethics committee approval for the study was obtained from the Necmettin Erbakan University KONÜDAM Experimental Medicine Application and Research Center Directorate with the decision number 2024-62 dated 11.07.2024.

2.4. Experimental Design

Wistar albino rats were randomly divided into 5 groups, each containing 7 rats (Table 1). MRN [11] and PCM [12] doses were determined from previous studies.

Table 1. Groups and experimental design

Group	Substance(s) Administered	Dose	Duration of Administration
Control (C)	Saline	1 ml	Once daily for 6 days (oral)
Morin (MRN)	Morin	100 mg/kg	Once daily for 6 days (oral)
Paracetamol (PCM)	Saline + Paracetamol	Saline: 6 days, PCM: 1000 mg/kg	PCM on day 6, 30 min after saline (oral)
PCM +MRN50	Paracetamol + Morin	PCM: 1000 mg/kg; MRN: 50 mg/kg	MRN: once daily for 6 days; PCM on day 6, 30 min later
PCM+MRN100	Paracetamol + Morin	PCM: 1000 mg/kg; MRN: 100 mg/kg	MRN: once daily for 6 days; PCM on day 6, 30 min later

On the seventh day, 24 hours after the final treatment, the animals were euthanized via decapitation under mild sevoflurane anesthesia. Blood samples were collected into anticoagulant-free vacuum tubes for biochemical evaluations. These samples were centrifuged at 3000 rpm for 10 minutes at +4 °C, and the resulting sera were separated. Portions of the kidney tissue were frozen at –20 °C for biochemical investigations, while other portions were fixed in 10% formaldehyde solution for subsequent histopathological examination.

2.5. Serum Renal Function Markers

Serum urea and creatinine concentrations were determined using commercially available assay kits (Diasis Diagnostic Systems, Istanbul, Turkey) following the protocols provided by the manufacturer.

2.6. Oxidative Stress Parameters

Kidney tissue was homogenized with 1.15% potassium chloride in a tissue homogenizer (IKA T18 digital ultra-

turrax, Germany). Glutathione peroxidase (GPx) was determined by Matkovics [13], glutathione (GSH) by Sedlak and Lindsay [14], malondialdehyde (MDA) by Placer et al. [15], superoxide dismutase (SOD) by Sun et al. [16], catalase (CAT) by Aebi [17] and protein determination by Lowry et al. [18] methods.

2.7. Real Time PCR (RT-PCR)

At the end of the experiment, mRNA transcription levels of genes listed in Table 2 were analyzed by the RT-PCR method in kidney tissues obtained. RNA isolation was performed using commercially available QIAzol Lysis Reagent (Qiagen, 79306). Isolated total RNA was converted to cDNA with OneScript Plus cDNA Synthesis Kit (ABM, G236, Richmond, Canada). Then, the PCR mixture was prepared by adding BlasTaq™ 2X qPCR MasterMix (ABM, G891, Richmond, Canada) with primer sequences, and the reaction started. Primers based on the Rattus norvegicus sequence were designed in the Oligo 6.0 primer design program. The procedures were carried out in appropriate temperature cycles in the Rotor-Gene Q (Qiagen) device according to the protocol specified by the manufacturer. Gene expressions obtained from the analysis were normalized with the β-Actin reference gene and evaluated using the 2-ΔΔCT method [19].

Table 2. Primer sequences

Gene	Sequences (5'-3')
NF-κB	F: AGTCCCGCCCCTTCTAAAAC R: CAATGGCCTCTGTGTAGCCC
TNF-α	F: CTCGAGTGACAAGCCCGTAG R: ATCTGCTGGTACCACCAGTT
Caspase-3	F: ACTGGAATGTCAGCTCGCAA R: GCAGTAGTCGCCTCTGAAGA
Bax	F: TTTCATCCAGGATCGAGCAG R: AATCATCCTCTGCAGCTCCA
Bcl-2	F: GACTTTGCAGAGATGTCCAG R: TCAGGTACTCAGTCATCCAC
KIM1	F:TGGCACTGTGACATCCTCAGA R: GCAACGGACATGCCAACATA
AQP2	F: AGCTGCCTTCTATGTGGCT R: GCGTTGTTGTGGAGAGCATT
β-Actin	F: CAGCCTTCCTTCTTGGGTATG R: AGCTCAGTAACAGTCCGCCT

2.8. Histological Analysis

Kidney tissues obtained from experimental groups were first kept in 10% neutral formalin solution for 24 hours as a fixative to be examined under a light microscope. According to routine paraffin follow-up procedures [20], tissues were dehydrated by passing through increasing grades of alcohol and cleared in xylene. Then, paraffin blocks were obtained from tissues treated with liquid paraffin. 5-micron sections were taken from paraffin blocks with a microtome and stained with hematoxylin-eosin (H&E). A binocular Olympus Cx43 light microscope (Olympus Inc., Tokyo, Japan) and an EP50 camera (Olympus Inc., Tokyo, Japan) were used to obtain images from stained preparations, and evaluation was performed using a blind method.

2.9. Statistical Analysis:

Group comparisons and statistical significance were evaluated using one-way ANOVA followed by Tukey's post hoc test (SPSS version 20.0; Chicago, IL, USA). A p-value of less than 0.05 was considered statistically significant. Data are presented as mean ± standard deviation (SD).

3. RESULTS

3.1. Kidney Function Tests

The effects of PCM and MRN applications on serum urea (Figure 1A) and creatinine (Figure 1B) levels were examined. According to the obtained data, it was determined that there was no difference between the Control and MRN groups ($p>0.05$), PCM application increased serum urea and creatinine levels compared to the Control and MRN groups ($p<0.001$), and MRN applied together with PCM was effective at doses of 50 and 100 and brought these increased values closer to the control group levels ($p<0.001$).

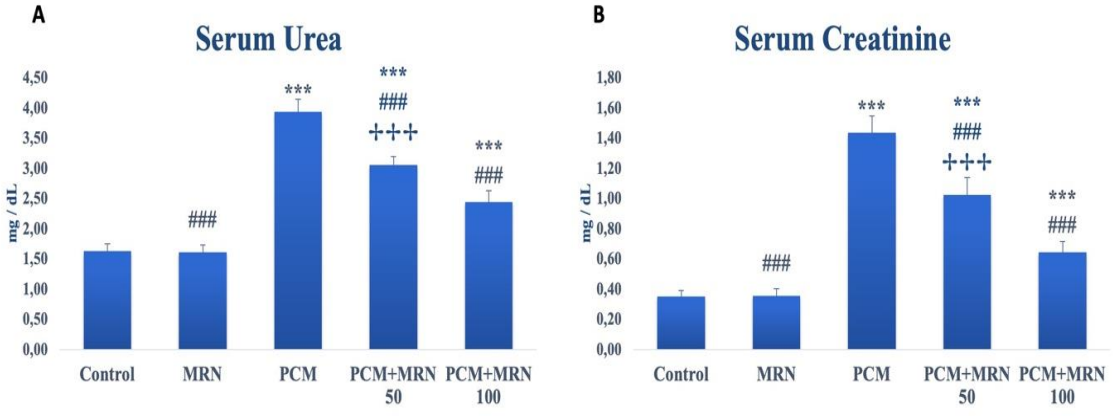

Figure 1. Effects of PCM and MRN on serum urea (A) and serum creatinine (B) levels. Values are given as mean ± SD. Control vs others: *p < 0.05, **p < 0.01, ***p < 0.001, PCM vs others: #p < 0.05, ##p < 0.01, ###p < 0.001, PCM+MRN50 vs PCM+MRN100: + p< 0.05, ++p < 0.01, +++p < 0.001.

3.2. Oxidative Stress Markers

The effects of PCM and MRN applications on kidney tissue oxidative stress markers MDA (Figure 2A), GSH (Figure 2B), SOD (Figure 2C), CAT (Figure 2D) and GPx (Figure 2E) were examined. According to the results, no difference was found in all parameters between the control and MRN groups ($p>0.05$). However, it was determined that PCM application increased MDA levels approximately 2-fold compared to the control group ($p<0.001$) and significantly decreased GSH levels and SOD, CAT and GPx activities ($p<0.001$). It was determined that MRN application, together with PCM, was effective in both doses, reduced PCM-induced increased MDA levels ($p<0.001$), and was effective in reducing oxidative stress by providing an increase in antioxidant parameters ($p<0.001$).

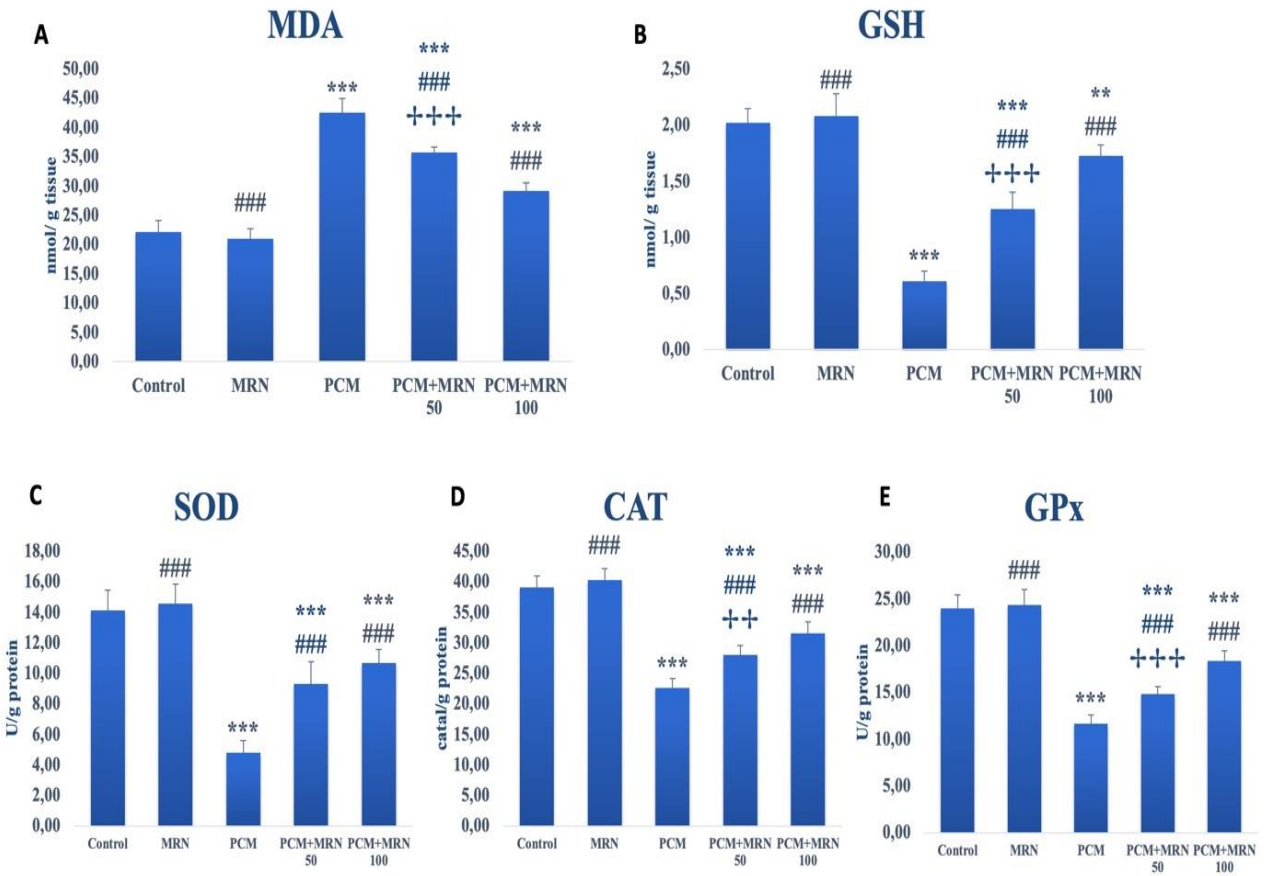

Figure 2. Effects of PCM and MRN on MDA (A) and GSH (B) levels and SOD (C), CAT (D), and GPx (E) activities in rat kidney tissues. Values are given as mean ± SD. Control vs others: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, PCM vs others: #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, PCM+MRN50 vs PCM+MRN100: ✢ $p< 0.05$, ✢✢$p < 0.01$, ✢✢✢$p < 0.001$.

3.3. Inflammation Results

When the mRNA expression levels of NF-κB (Figure 3A) and TNF-α (Figure 3B) markers of inflammation were examined, it was determined that PCM application caused an increase in both parameters compared to the control and MRN groups and accelerated inflammation ($p<0.001$), and that the application of MRN 50 (NF-κB: $p<0.01$ and TNF-α: $p<0.001$) and MRN 100 ($p<0.001$) doses together with PCM was effective in suppressing PCM-induced increased inflammation by reducing NF-κB and TNF-α expression levels.

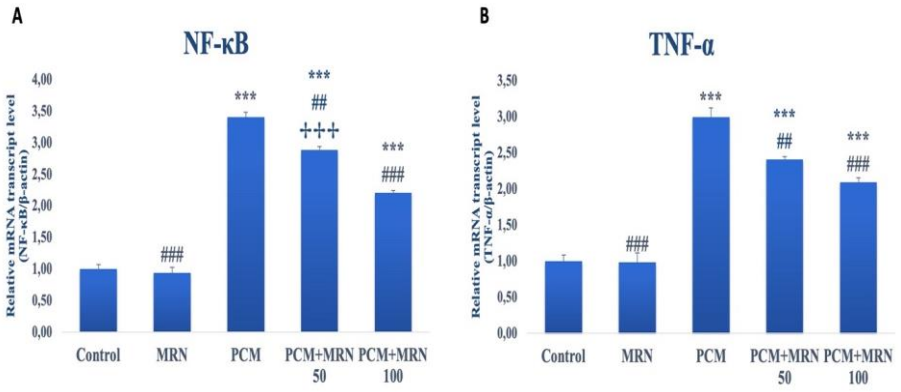

Figure 3. Effects of PCM and MRN on NF-κB (A) and TNF-α (B) mRNA transcription levels in rat kidney tissues. Values are given as mean ± SD. Control vs others: *p < 0.05, **p < 0.01, ***p < 0.001, PCM vs others: #p < 0.05, ##p < 0.01, ###p < 0.001, PCM+MRN50 vs PCM+MRN100: ✢ p< 0.05, ✢✢p < 0.01, ✢✢✢p < 0.001.

3.4. Apoptosis Results

The effects of PCM and MRN applications on the mRNA expression of apoptotic markers Caspase-3 (Figure 4A) and Bax (Figure 4B) and antiapoptotic marker Bcl-2 (Figure 4C) were examined. According to the data obtained, it was determined that PCM application increased the expressions of Caspase-3 and Bax compared to the control and MRN groups, while suppressing the expression of Bcl-2 and triggering the apoptotic process ($p<0.001$). It was determined that the MRN application together with PCM was effective in suppressing apoptosis by decreasing the expressions of Caspase-3 (MRN50: $p<0.01$; MRN100: $p<0.001$) and Bax (MRN50 and MRN100: $p<0.001$) and increasing the expression of Bcl-2 (MRN100: $p<0.01$).

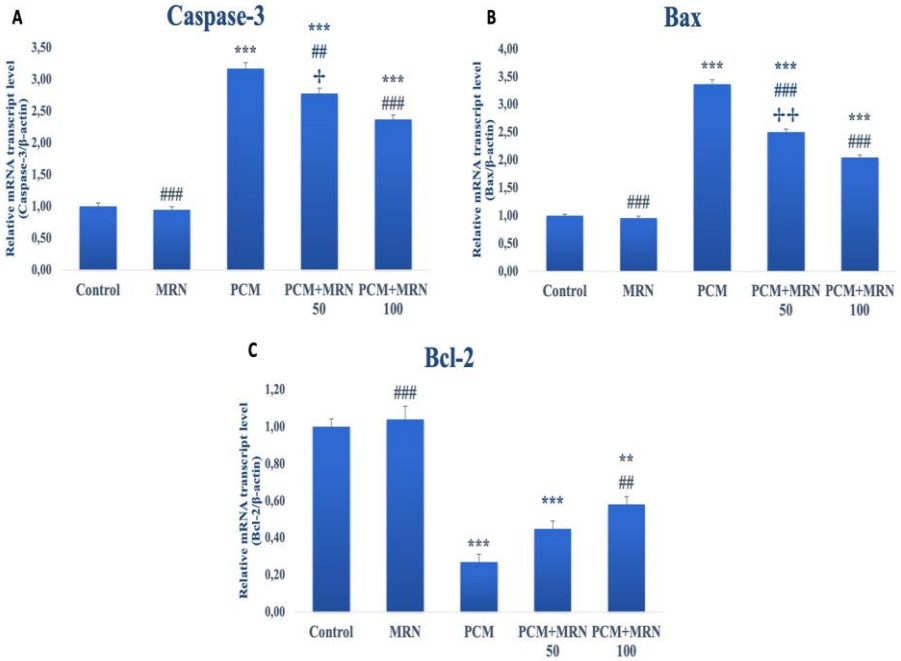

Figure 4. Effects of PCM and MRN on Caspase-3 (A), Bax (B), and Bcl-2 (C) mRNA transcription levels in rat kidney tissues. Values are given as mean ± SD. Control vs others: *p < 0.05, **p < 0.01, ***p < 0.001, PCM vs others: #p < 0.05, ##p < 0.01, ###p < 0.001, PCM+MRN50 vs PCM+MRN100: ✢ p< 0.05, ✢✢p < 0.01, ✢✢✢p < 0.001.

3.5. Kidney Damage Markers

Kidney damage markers Kidney Injury Molecules 1 (KIM1) and Aquaporin 2 (AQP2) mRNA expression levels were examined. Accordingly, it was determined that PCM administration increased kidney KIM1 (Figure 5A) levels and decreased AQP2 (Figure 5B) levels ($p<0.001$) compared to the control group and caused kidney damage. It was determined that the combined administration of PCM and MRN decreased KIM1 levels and increased AQP2 levels (MRN50: $p<0.05$; MRN100: $p<0.001$).

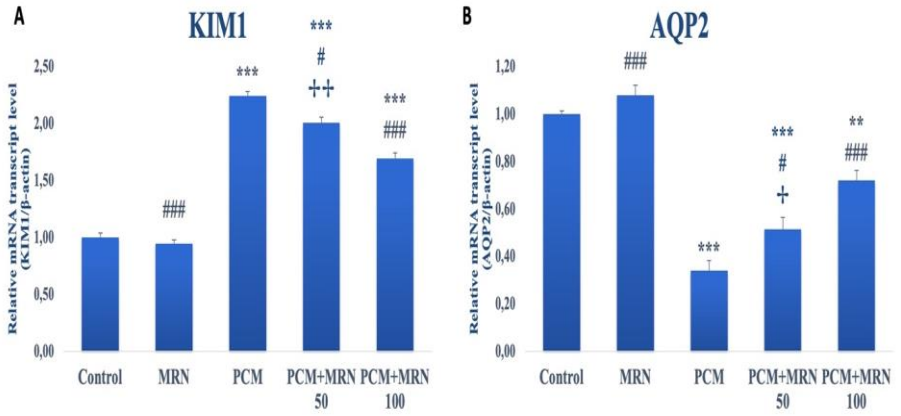

Figure 5. Effects of PCM and MRN on KIM1 (A) and AQP2 (B) mRNA transcription levels in rat kidney tissues. Values are given as mean ± SD. Control vs others: *p < 0.05, **p < 0.01, ***p < 0.001, PCM vs others: #p < 0.05, ##p < 0.01, ###p < 0.001, PCM+MRN50 vs PCM+MRN100: ✢ p< 0.05, ✢✢p < 0.01, ✢✢✢p < 0.001.

3.6. Histopathological Results

Kidney histological images of different groups are presented in Figure 1. When the images of kidney tissues of control and MRN groups were examined, they exhibited well-organized and normal histological features (Figure 6a, 6b). In contrast, atrophic glomerular structures and increased Bowman spaces were noted in the kidneys of rats treated with PCM. In addition, epithelial cells of many cortical tubule structures were shed, and there were degenerative changes and pyknotic nuclei in places. The PCM application caused mononuclear inflammatory cell infiltrates in the interstitial area and vascular congestion (Figure 6c). However, simultaneous PCM+MRN treatments significantly improved PCM-induced pathological changes in kidney tissues. Vascular congestion and tubular degeneration were mild in these groups (Figure 6d, 6e).

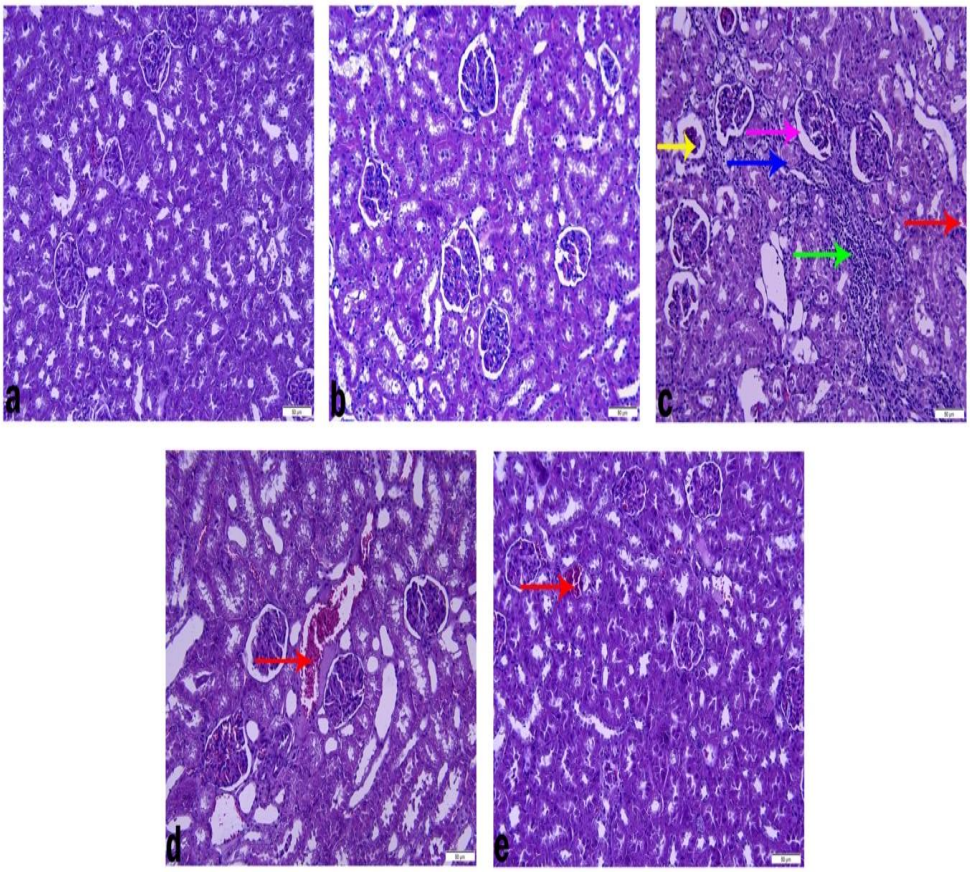

Figure 6. Representative images showing the histopathological effects of PCM and MRN on kidney tissue. a: Normal control group, b: MRN group, c: PCM group, d: PCM+MRN 50, and e: PCM+MRN 100 groups. In the images, yellow arrow: atrophic glomeruli, green arrow: mononuclear inflammatory cell infiltrates, blue arrow: cortical tubule cell degeneration, red arrow: vascular congestion, pink arrow: increased Bowman space. (H&E, x 200).

4. DISCUSSION AND CONCLUSION

PCM is a painkiller and antipyretic drug that can be obtained without a prescription and is safe and effective at normal therapeutic doses, but causes renal toxicity at high doses [21]. High dose PCM administration increases MDA levels by creating oxidative stress in the kidney tissue of rats and weakens antioxidant capacity by decreasing SOD, GSH and GPx levels belonging to the antioxidant defense system. It is also known to impair renal functions by increasing serum creatinine and urea levels [22]. The present study aimed to investigate the effects of MRN, a natural flavonoid, on PCM-induced renal damage.

The most important side effect of PCM is that it causes nephrotic damage by weakening glomerular function. Creatinine is produced from protein metabolism in the muscles, and most of the creatinine is filtered from the blood by the kidneys and excreted in the urine. In kidney disease, the rate of urea production exceeds the clearance rate, which causes an increase in the amount of serum urea [23]. It has been shown in different studies that PCM application causes serious damage to the kidney and impairs its functions [2,24]. In the presented study, it was determined that PCM application caused an increase in serum urea and creatinine levels and that the probable cause of this increase was the decrease in glomerular filtration. It was determined that the MRN application, together with PCM, was effective in reversing these parameters and reducing serum urea and creatinine levels. It has been reported in different studies that MRN regulates glomerular filtration in kidney toxicities caused by different chemical agents and helps to maintain serum urea and creatinine levels at normal levels [10,25,26].

Oxidative stress and free radicals play a role in the pathogenesis of paracetamol-induced renal injury [27]. Free radicals increase lipid peroxidation and trigger the release of inflammatory mediators, leading to renal tissue damage [28]. MDA, a byproduct of lipid peroxidation, serves as an indicator of tissue injury caused by various chain reactions [29,30]. Elevated lipid peroxidation levels and reduced activity of antioxidant enzymes are recognized as key contributors to oxidative stress in renal tissue [31,32]. Yousef et al. [33] reported that PCM increases lipid peroxidation while decreasing antioxidant enzyme activities, thus developing oxidative stress in renal tissue. The body has developed various defense mechanisms against oxidative stress, including antioxidant enzymes such as SOD, CAT, GPx, and the non-enzymatic antioxidant GSH [34]. Cells subjected to oxidative stress are safeguarded by both enzymatic and non-enzymatic antioxidant defense systems, which play a crucial role in maintaining cellular integrity [35,36]. GSH, a non-enzymatic tripeptide, plays a vital role in defending tissues and organs against the harmful impact of ROS. Within the cytochrome P450 pathway, GSH detoxifies NAPQI, a reactive metabolite of paracetamol. However, when GSH reserves are exhausted, excess NAPQI interacts with cellular protein macromolecules, triggering cellular damage that can result in necrosis or apoptosis [37]. While SOD converts superoxide radical to molecular oxygen and hydrogen peroxide, CAT converts hydrogen peroxide to water and molecular oxygen. GPx, similar to CAT, is responsible for the detoxification of hydrogen peroxide [38]. In the presented study, it was determined that PCM increased MDA levels, and in addition, decreased GSH levels and SOD; CAT and GPx activities, causing oxidative stress in the kidney tissue. It was determined that MRN given with PCM was effective in suppressing oxidative stress by regulating these parameters inversely at both doses. It has been demonstrated in different studies that morin is effective in suppressing lipid peroxidation thanks to its antioxidant properties, increases antioxidant enzyme activities, and therefore protects the tissue from oxidative stress [6,7,9].

Growing evidence indicates that oxidative stress plays a crucial role in promoting and sustaining the inflammatory response [39]. Inflammation is one of the adaptive responses to various insults involving biological and chemical agents [40]. The increase in ROS triggers inflammation by elevating NF-κB and TNF-α levels [41,42]. NF-κB, a transcription factor, migrates from the cytosol to the nucleus after dissociating from IκB, where it regulates the expression of up to 500 genes, including those that promote proinflammatory cytokines like TNF-α [43]. Therefore, inhibition of NF-κB is important for the suppression of inflammation [44]. El-Boshy et al. [45] reported that PCM increases oxidative stress by increasing ROS levels in kidney tissue, and that the developing oxidative stress accelerates inflammation by increasing inflammatory cytokines such as NF-κB and TNF-α. In the presented study, it was determined that PCM increased the levels of NF-κB and TNF-α in kidney tissue; this increase developed in parallel with oxidative stress and accelerated the inflammation process in kidney tissue. In addition, it was determined that the MRN application was effective in suppressing PCM-induced inflammation by reducing NF-κB and TNF-α levels. Studies have shown that MRN has anti-inflammatory properties and is effective in suppressing inflammation [6,10,26].

Apoptosis, which eliminates damaged or dangerous cells in the body, also damages healthy cells with the development of oxidative stress [46,47]. Caspase-9 is activated by the combination of cytochrome c and Apaf-1 [48]. The activated caspase-9 activates caspase-3, known as the executioner caspase, and thus the apoptotic process begins. While Bax is an apoptotic factor that opens membrane pores in the mitochondria, Bcl-2 shows an antiapoptotic effect by preventing the opening of mitochondrial membrane pores [49,50]. El-Bakry et al. [51] and Aktaş-Şenocak et al. [52] reported that PCM induces apoptosis through caspase-3 activation. Similarly, Ahmad et al. [27] found that Bcl-2 levels decreased and Bax levels increased in rats to which PCM was applied, thus accelerating the apoptotic process of the cell. In the presented study, it was determined that caspase-3 and bax expression levels increased in the kidney tissue of rats to which PCM was applied, antiapoptotic Bcl-2 expression decreased, and the apoptotic process of the kidney tissue accelerated, and this acceleration in the apoptotic process was parallel to oxidative stress and inflammation. It was

determined that the MRN application together with PCM was effective in suppressing apoptosis by increasing Bcl-2 expression and decreasing caspase-3 and Bax expressions, and MRN showed antiapoptotic properties. In different chemical toxicity models, it has been shown that MRN is effective in suppressing apoptosis, suppressing caspase-3 and bax expressions, and increasing Bcl-2 expression [6,9,11].

When the data obtained in the study were evaluated as a whole, it was determined that PCM application caused damage to the kidney tissue by stimulating oxidative stress, inflammation and apoptosis, and that MRN supportive treatment was effective in protecting the kidney tissue from PCM-induced damage by reversing these pathways, and that MRN application would be beneficial in PCM-induced kidney toxicities.

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