The Efficacy of Mumijo Extract in Protecting Against Amoxicillin/Clavulanate-Induced Apoptotic Damage in Renal Tissues of Rats

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Keywords. Amoxicillin/Clavulanate; Mumijo extract; Oxidative stress; Renal apoptosis; Antioxidant therapy; Bax/Bcl-2 proteins

Introduction. Amoxicillin/Clavulanate (A/C) is a widely used antibiotic that can induce oxidative stress and renal apoptosis through reactive oxygen species (ROS). Mumijo extract (FA), known for its antioxidant and anti-inflammatory properties, has shown promise in mitigating such damage. This study aims to evaluate the efficacy of FA in protecting against A/C induced renal tissue damage in rats.

Methods. The study included 28 Wistar albino rats, divided into four groups: Sham (saline), A/C (10 mg/kg/day), FA (100 mg/kg/day), and A/C+FA. A/C and FA were administered orally for 21 days. Renal function markers (urea, creatinine), oxidative stress parameters (TAS, TOS, MDA), and histopathological findings were analyzed. Bax and Bcl-2 protein expressions were assessed to evaluate apoptosis.

Results. A/C administration significantly increased renal urea, creatinine, TOS, and MDA levels while reducing TAS ($P < .05$). Histopathological analysis revealed tubular dilatation, degeneration, and inflammation in the A/C group. FA supplementation in the A/C+FA group significantly reduced oxidative stress markers and improved TAS levels, histopathological scores, and apoptosis indicators (increased Bcl-2 and reduced Bax expressions). FA alone demonstrated no adverse effects on renal parameters.

Conclusion. FA effectively mitigates A/C-induced oxidative stress and renal apoptosis, preserving tissue integrity and function. These findings suggest FA as a potential therapeutic agent for managing drug-induced nephrotoxicity.

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INTRODUCTION

Amoxicillin/Clavulanate (A/C) is a broad-spectrum antibiotic combination consisting of amoxicillin, a β-lactam antibiotic, and potassium clavulanate, a β-lactamase enzyme inhibitor.[1] Since amoxicillin is susceptible to degradation by β-lactamase enzymes produced by resistant bacteria, it is used in combination with clavulanic acid.[2] It is commonly preferred for the treatment of sinusitis, otitis, bacterial bronchitis, pneumonia, and infections caused by antibiotic-resistant bacteria.[3] A/C is primarily excreted via the kidneys.[4] Studies have shown that A/C may cause renal damage by increasing oxidative stress.[5] High-dose A/C applications may cause nephritis, oliguric renal failure, and acute kidney injury in advanced stages.[6] Increased oxidative stress leads to the accumulation of reactive oxygen species (ROS) in cells. ROS react with unsaturated fatty acid chains, inducing lipid peroxidation and resulting in the formation of toxic

byproducts such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal.[7] Additionally, ROS plays a significant role in triggering the apoptosis process. Antioxidant enzymes, on the other hand, serve as a critical defense mechanism in preventing oxidative stress and apoptosis.[8,9]

Apoptosis is a form of programmed cell death in an organism. The term "apoptosis," first described in the 1970s, has been the subject of substantial advancements to date.[10] Apoptosis occurs through two distinct upstream pathways, either intrinsic or extrinsic.[11] The ratio of Bax protein to B-cell lymphoma 2 (Bcl-2) protein is a key regulator that determines the cell's susceptibility to apoptosis. Bax protein promotes apoptosis, while Bcl-2 exerts an anti-apoptotic effect.[12,13]

Mumijo, also known by various other names such as shilajit, salajeet, and mummiyo, is a semi-solid, blackish-brown substance with a distinct odor that forms through the long term phytomineral diffusion over many years in rock formations.[14,15] Its chemical composition is well-characterized and is reported to contains 60-70% humus.[16] Fulvic acid (FA) is the main biologically active component of this mixture.[17,18] Preclinical studies have demonstrated that FA exhibits a wide range of biological activities, including antioxidant, anti-inflammatory, anti-apoptotic, and mitochondrial protective properties.[19] Additionally, it has been shown to have the capacity to induce apoptosis in human breast cancer cells and in alcohol-independent liver damage.[20,21] Moreover, it has been reported to prevent liver damage in non-alcoholic fatty liver disease (NAFLD) induced by a high-fat diet and to induce apoptosis in hepatic cancer cells.[22,23]

MATERIALS AND METHODS

Determination of Amoxicillin-Clavulanate Dosage

Separate Augmentin-BID® tablets (GlaxoSmithKline, Brantford, UK; 875 mg amoxicillin/125 mg clavulanic acid) were prepared for each rat. The tablets were dissolved in distilled water to form a solution. It was administered orally via gavage at a dose of 10 mg/kg/day for 21 days.[24]

Determination of Fulvic Acid Dosage

Fulvic acid used in the study was administered orally via gavage at a dose of 100 mg/kg/day. The dose selection was based on previous preclinical studies and its efficacy and safety have been confirmed in rodent models.[25]

Determination of Experimental Groups

All procedures were conducted in accordance with the guidelines of the Laboratory Animal Care and Use Manual.[26] A total of 28 Wistar albino rats, aged 8–10 weeks, were included in the study and randomly divided into four groups (n = 7 in each group). Rats were housed under standard conditions (12-hour light/dark cycle, 55–65% humidity, and a temperature of 22 ± 2°C) with unrestricted access to food and water.

- **Group I (Sham group):** Rats received 1 cc of normal saline for 21 days.
- **Group II (A/C group):** Rats received amoxicillin-clavulanate at a dose of 10 mg/kg/day for 21 days.
- **Group III (FA group):** Rats were administered FA at a dose of 100 mg/kg/day once daily via oral gavage for 21 days.
- **Group IV (A/C+FA group):** Rats received both treatments for 21 days.

The study was terminated on day 21. All treatments were administered orally via gavage. General anesthesia was achieved with intraperitoneal administration of ketamine HCl (90 mg/kg; Ketalar, Pfizer Inc, USA) and xylazine HCl (10 mg/kg; Rompun, Bayer Health Care AG, Germany). Following anesthesia, blood samples were collected via cardiac puncture, and the animals were euthanized. Kidney tissues and blood samples were collected. All waste materials were disposed of according to medical waste protocols. Although baseline TAS, TOS, MDA, Bax, Bcl-2, Cr and BUN values were not measured before the interventions, the random allocation of animals into homogeneous groups with similar age and weight profiles was assumed to provide comparable baseline levels.

Biochemical Analysis

Blood samples collected from the heart were placed in tubes containing gel clot activator and separators, followed by centrifugation at 3000 RPM for eight minutes. Plasma samples were used to measure urea and creatinine levels using ELISA kits. Results were expressed in mg/dL.[27,28]

Total Antioxidant Status (TAS) Measurement

Serum total antioxidant status was measured using kits provided by Rel Assay Diagnostics (Gaziantep, Turkey) based on a method developed by Erel. TAS values were analyzed spectrophotometrically using an automatic analyzer (AU5800; Beckman Coulter, Inc., Brea, CA, USA) with a colorimetric test kit having a coefficient of variation of 10% and a linear range of 0–2.75 mmol/L.[29]

Total Oxidant Status (TOS) Measurement

Serum total oxidant status was measured using kits provided by Rel Assay Diagnostics (Gaziantep, Turkey) according to the method developed by Erel.[30] TOS values were analyzed on an automated analyzer (AU5800; Beckman Coulter, Inc., Brea, CA, USA) using a colorimetric test kit reported with a 10% coefficient of variation and a linear measurement range of 0–33.5 mmol/L. Results were expressed as μmol H_2O_2 Equivalent/L.

Malondialdehyde (MDA) Analysis

Malondialdehyde (MDA), a byproduct of lipid peroxidation, was measured using rat-specific ELISA kits provided by Bioassay Technology Laboratory (Shanghai, China). Results were reported in nmol/L.[31]

Histopathological Examination

After anesthesia with ketamine HCl (90 mg/kg) and xylazine HCl (10 mg/kg), kidney tissues were fixed in 10% formalin and sent to the histology laboratory. Samples were kept in formalin for at least 72 hours, followed by washing in tap water for 12 hours. The tissues underwent routine histological processing, and 5-μm-thick sections were stained with H&E. Sections were examined under a light microscope (Zeiss microscope, Germany) and scored for histopathological damage. Damage parameters included cellular vacuolization, congestion, tubular degeneration, inflammation, and tubular dilatation, scored on a scale of 0 to 3.[32-34]

Bax and Bcl-2 Expression

Kidney tissues were subjected to routine histological procedures for immunostaining, then washed with phosphate-buffered saline (PBS) and incubated with 3% hydrogen peroxide (Thermo Fisher, Fremont, CA, USA) for 20 minutes before blocking solution was applied for 8 minutes. Then, it was incubated at a 1:100 dilution with Bcl-2 and Bax primary antibodies (catalog numbers: sc-65891 and sc-7480, Santa Cruz Biotechnology Inc). DAB chromogen was used to visualize the expression. Harris was stained with hematoxylin for contrast and imaged the next day under a Zeiss Imager A2 light microscope. Expression intensity was assessed by a blinded histologist and scored as follows: 0 = no brown-stained cells, 1 = low, 2 = moderate, 3 = strong.[35]

All biochemical analyses and histopathological evaluations were performed by investigators who were blinded to group allocation to ensure objectivity and minimize bias.

Statistical Analysis

Statistical analyses were performed using v27.0 (IBM Corp., Armonk, NY, USA). The assumption of normal distribution of the data was assessed using the Shapiro-Wilk test Data showing a normal distribution were tested using ANOVA for group comparisons, followed by Tukey's test for post-hoc analysis. Data that did not follow a normal distribution were analyzed using the Kruskal-Wallis H test followed by Mann-Whitney U pairwise comparisons. Values are expressed as mean ± standard deviation. Those with a P-value < .05 were considered statistically significant.

Ethics Approval and Consent

All procedures included in the experimental protocols were approved by the Animal Ethics Committee of Dicle University for Experimental Animals (Protocol No: 25/03/2024-680818, Diyarbakır, Turkey).

RESULTS

Biochemical Analyses

Urea and Creatinine Analysis

The mean ± standard deviation (SD) values of urea and creatinine levels in the Sham, A/C, FA, and A/C+FA groups are presented in Table 1. The lowest levels of both urea and creatinine were observed in the FA group (46.28 ± 6.62, 0.50 ± 0.11), whereas the highest levels were found in the A/C group (134.71 ± 24.60, 2.55 ± 1.01). Intergroup comparisons showed no significant differences in urea and creatinine levels between the Sham and FA groups (P = .949, P = .898). However, a significant difference was observed between the

Table 1. Comparison of Urea, Creatinine, MDA, TAS and TOS Values between Groups

Groups	Urea (mg/dl)	Creatinine (mg/dl)	TAS (mmol/L)	TOS (μmol/L)	MDA (nmol/L)
Sham	47.57 ± 3.59**	0.51 ± 0.07**	1.19 ± 1.46	44.90 ± 10.84**	1.17 ± 0.11**
A/C	134.71 ± 24.60*,**	2.55 ± 1.01*,**	1.14 ± 0.08**	130.50 ± 17.03*,**	1.80 ± 0.20*,**
FA	46.28 ± 6.62	0.50 ± 0.11	1.39 ± 0.14	41.92 ± 5.82	1.15 ± 0.09
A/C+FA	89.14 ± 14.43**	1.50 ± 0.60*	1.38 ± 0.09**	109.43 ± 13.92*	1.46 ± 0.10*,**

A/C: Amoxicillin/Clavunate, FA: Mumijo extract, MDA: Malondialdehyde, TAS: Total antioxidant status, TOS: Total oxidant status. **Comparing Sham and A/C groups ($P < .005$), *Comparing A/C and A/C+FA groups ($P < .05$).

A/C and A/C+FA groups, indicating that kidney function tests improved in the A/C+FA group, with reduced urea and creatinine levels due to the effect of FA.

TAS Analysis

The mean ± SD values of total antioxidant status (TAS) in the Sham, A/C, FA, and A/C+FA groups are shown in Table 1, there was no significant difference in TAS levels between the Sham and AC groups ($P = .654$). However, TAS levels in the Sham group were significantly lower than those in the FA group ($P = .025$), with the highest mean antioxidant level observed in the FA group (1.39 ± 0.14).

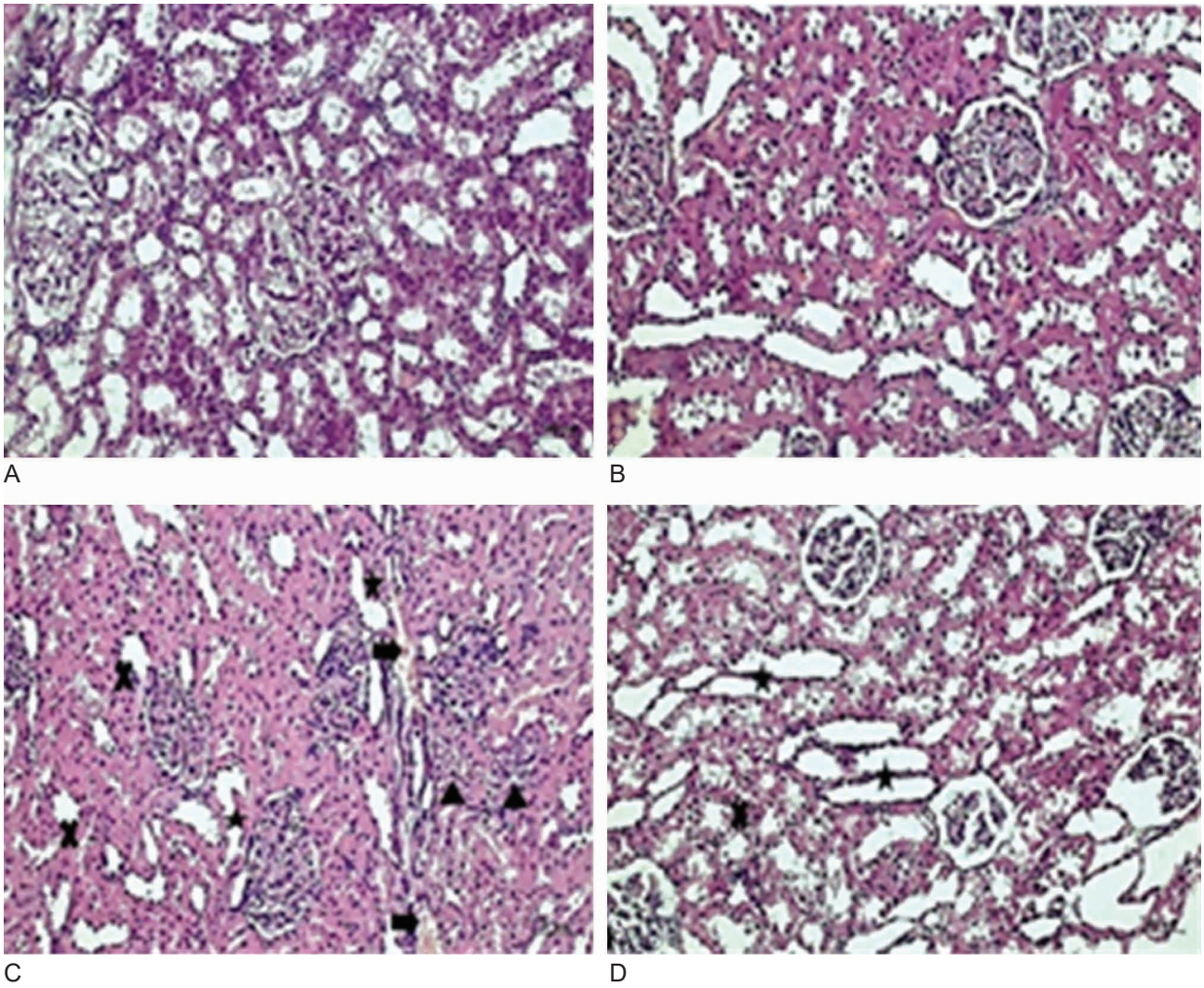

Figure 1. Light microscope images of kidney tissues (Staining: H&E, Magnification: 20×). (A) Sham group; (B) FA group; (C) A/C group; (D) A/C + FA group. Congestion (arrow), inflammatory cell infiltration (triangle), degeneration and atrophy in distal tubules (star), dilation in proximal tubules (cross).

TOS Analysis

The mean ± SD values of total oxidant status (TOS) in the Sham, A/C, FA, and A/C+FA groups are detailed in Table 1. Statistical comparisons showed no significant difference in TOS levels between the Sham and FA groups (P = .749) (Table1). The highest mean TOS level was found in the A/C group (130.50 ± 17.03). A significant reduction in TOS levels was observed in the A/C+FA group compared to the AC group (P = .035), suggesting that FA ameliorated oxidative stress in the animals.

MDA Analysis

It was determined that the MDA level, which rose to 1.80 ± 0.20 nmol/mL in the A/C group, decreased to 1.46 ± 0.10 nmol/mL in the A/C+FA group, and that this decrease was statistically significant (P = .015). A decrease in MDA levels, the end product of oxidative stress, was detected in the group treated with FA (Table1).

Histopathological Findings

Under anesthesia, the animals were euthenized via exsanguination, and kidney tissues were collected. Hematoxylin and Eosin (H&E) staining of prepared slides revealed normal histological structures in the kidneys of the Sham and FA groups. In contrast, kidney tissues in the A/C group exhibited tubular dilatation in the cortex, degeneration in proximal and distal tubular epithelial cells, and degenerated epithelial cells in the medulla. In the A/C+FA group, histopathological findings were significantly improved, with reduced inflammation and fewer degenerative changes (Figure 1). Histological damage scores confirmed that the highest scores were observed in the A/C group (2.28 ± 0.48), while the A/C+FA group demonstrated significantly lower scores (0.71 ± 0.48) (P = .001) (Figure 2).

Bax and Bcl-2 Expression

Microscopic examination of kidney tissues revealed moderate positive (+) Bax expression in the A/C group. In the A/C+FA group, Bax expression was reduced, indicating a protective effect of FA against apoptosis. Additionally, the balance of Bcl-2 expression was maintained in the A/C+FA group, with an increase in positive (+) Bcl-2 expression. The most prominent Bcl-2 expression was observed in the FA group (Figure 3).

DISCUSSION

Amoxicillin/Clavulanate is a broad-spectrum and effective antibiotic commonly used in the treatment of respiratory tract infections and otitis media. It is considered one of the most effective oral agents against *Streptococcus pneumoniae* strains with moderate or high resistance to penicillin.[36] A study on the antimicrobial susceptibility of bacterial pathogens isolated from patients with

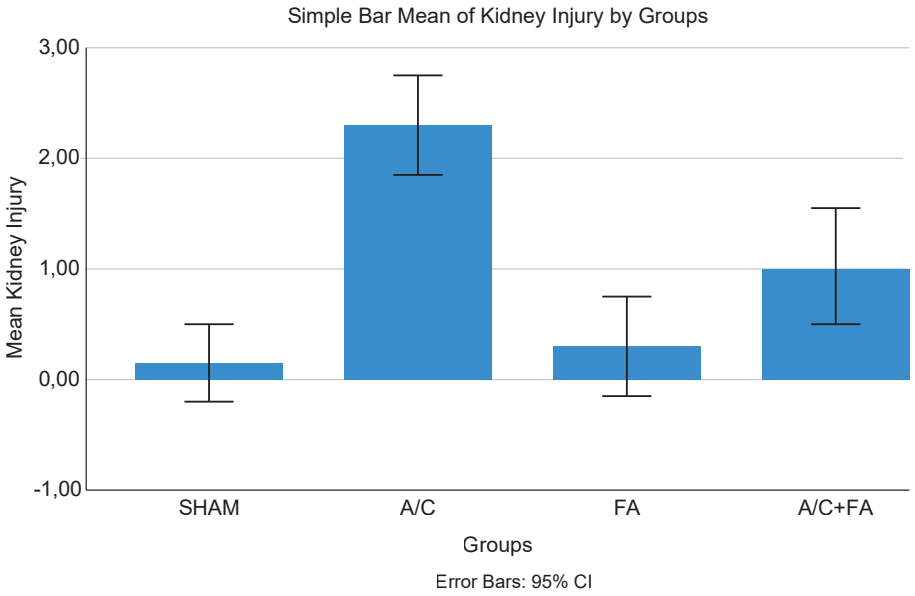

Figure 2. Statistical mean of kidney damage scores based on intergroup comparisons (AC: Amoxicillin/Clavulanate, FA: Fulvic acid).

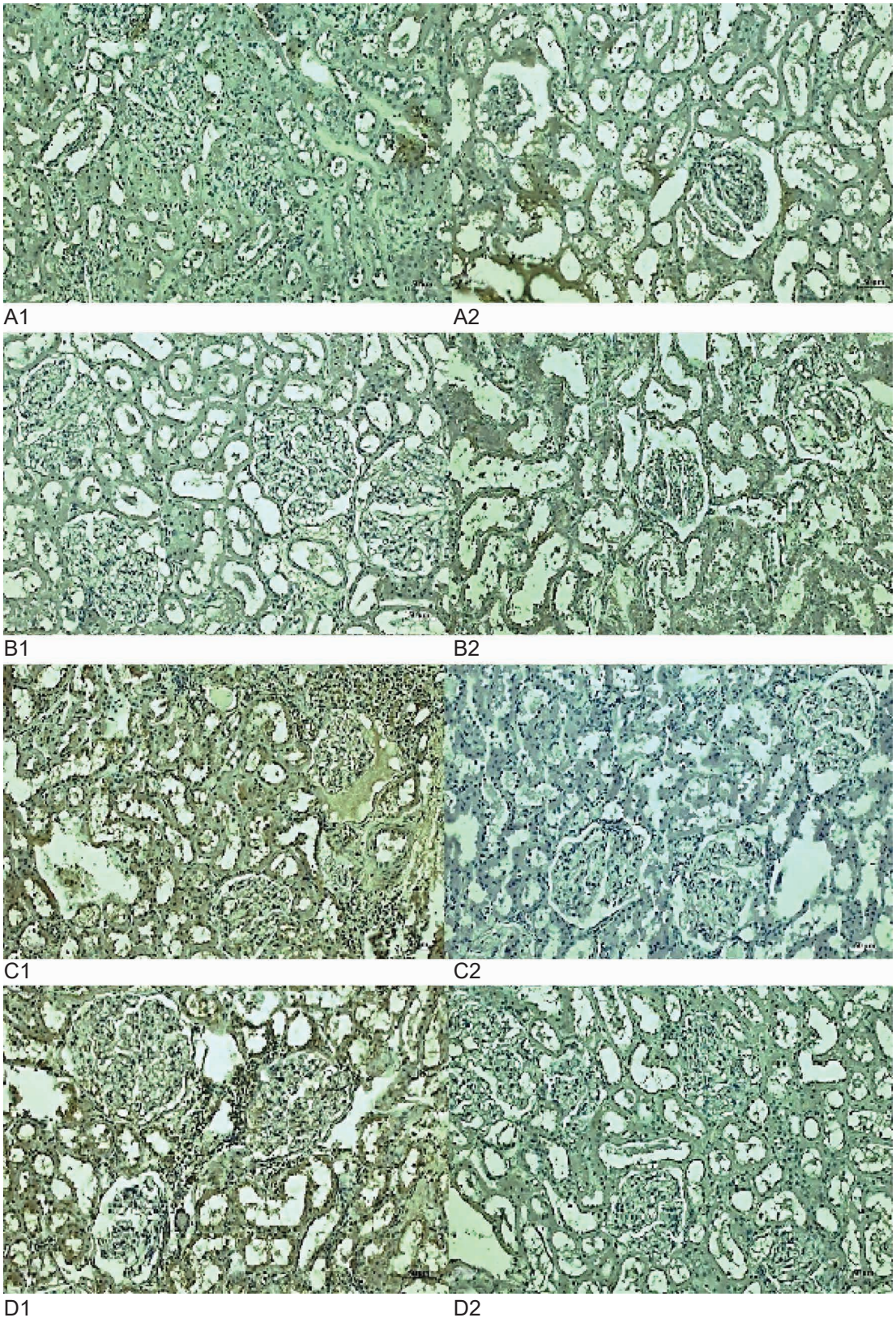

Figure 3. Light microscope images of kidney tissues. (A1) Sham group; (B1) FA group; (C1) A/C group; (D1) A/C+FA group, Staining; Bax, Counterstaining; Hematoxylin, Magnification; 20×. (A2) Sham group; (B2) FA group; (C2) A/C group; (D2) A/C+FA group, Staining; Bcl-2, Counterstaining; Hematoxylin, Magnification; 20×.

respiratory tract infections has confirmed the extent of bacterial resistance.[37]

Free radicals are molecules containing one or more unpaired electrons in their outer orbital.[38] In cells, many oxygen molecules are converted into water as a result of enzymatic activities. Some enzymes, however, leak electrons to oxygen molecules, leading to the formation of free radicals.[39] Free radicals react with phospholipid-unsaturated fatty acid residues, which are highly susceptible to oxidation, resulting in lipid peroxidation and subsequent formation of MDA. Elevated MDA levels exhibit carcinogenic effects in host cells.[40] Under normal circumstances, there is a balance between reactive oxygen species (ROS) and the antioxidant defense system.[41] However, when the balance shifts in favor of oxidants, it is termed oxidative stress. In cases where the organism's antioxidant defense system is insufficient, increased levels of free radicals cause cellular damage and functional impairments.[42]

Lucena *et al.* observed cytolytic damage in young individuals undergoing short-term A/C treatment, while older individuals experienced cholestatic/mixed-type damage during longer treatment durations.[43] It is well-documented that bactericidal antibiotics not only kill bacteria but also trigger the production of harmful reactive species. Dwyer *et al.* confirmed this phenomenon in studies showing that hydrogen peroxide could reach lethal levels during antibiotic treatment.[44] Kohanski *et al.* reported that three major classes of bactericidal antibiotics—aminoglycosides, quinolones, and β-lactams—induce lethal doses of hydroxyl radicals via the Fenton reaction, leading to cell death.[45] Bactericidal antibiotics not only kill bacteria but can also induce oxidative stress in cells by increasing the production of reactive oxygen species (ROS).[44,45] Under normal conditions, there is a balance between ROS and the antioxidant defense system;[46] however, A/C application disrupted this balance in favor of oxidants, increasing lipid peroxidation and MDA formation. In our study, the significantly lower total antioxidant capacity (TAS) values in the A/C group compared to the other groups revealed insufficient antioxidant defense and the severity of oxidative stress (Table 1). ROS increase-related obstruction, inflammation, and tubular degeneration in the kidneys, along with increased Bax protein expression, indicate that apoptotic pathways are activated.

In developed countries, numerous studies have highlighted the therapeutic effects of Mumijo on various diseases. It has been reported to have beneficial effects on gastrointestinal disorders,[47] as well as on bone pain, and fracture healing.[48] Additionally, it is known for its memory-enhancing, neuroprotective, anti-inflammatory, and antioxidant properties.[49,50] The biological effects of Mumijo are attributed to its content of di-benzo-alpha-pyrone, humic acid, and fulvic acid.[51] Studies have shown its reparative effects in regulating acid-pepsin secretion and reducing gastric ulcer index. Furthermore, it has been demonstrated to possess antioxidant activity, playing critical roles in cellular repair, regeneration, and wound healing.[52,53] In our study, FA was shown to contribute to renal tissue regeneration and cellular repair. Oral administration of FA at a daily dose of 100 mg/kg resulted in the reversal of histopathological changes in rats. The antioxidant effects reported in previous studies were corroborated in our study by the significantly higher serum TAS levels in the FA treated group compared to other groups. Ghezelbash and colleagues reported that FA prevented liver damage associated with a high-fat diet, while Pant and colleagues reported that it triggered an apoptotic response in cancer cells.[54,55] Jambi and Alshubaily reported that FA at doses of 150–250 mg/kg improved tubular atrophy and glomerular structures in kidney tissue and regulated urea and creatinine levels.[56]

Limitations

Our findings indicate that FA improves kidney function tests and, when administered at a dose of 100 mg/kg, positively affects the Bax/Bcl-2 ratio by suppressing inflammatory and apoptotic signals. This result supports the notion that FA has the potential to protect kidney tissue through both antioxidant and anti-apoptotic mechanisms. Furthermore, it was observed that FA limits ROS-induced cellular damage and the apoptotic process by increasing antioxidant capacity, and alleviates pathological changes such as oxidative stress and tubular degeneration caused by A/C toxicity. However, it is thought that FA may provide protective effects for kidney tissue against potential nephrotoxic agents such as A/C.

CONCLUSION

Biochemical, histopathological, and immunohistochemical analyses revealed that fulvic acid contributes to the improvement of impaired kidney function tests, restores oxidative balance by reducing increased oxidative stress, and demonstrates its anti-apoptotic potential by regulating the balance between Bax/Bcl-2 proteins. These findings suggest that fulvic acid may be a potential protective agent in preventing kidney failure. However, preclinical studies need to be expanded and advanced clinical trials need to be planned before moving to clinical applications.

CONFLICT OF INTEREST STATEMENT

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

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