



# Alamandine alleviates methotrexate-induced nephrotoxicity in rats by targeting oxidative stress and inflammation

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## Abstract

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**Aim:** Nephrotoxicity due to the use of methotrexate (Mtx) is one of the most important problems associated with chemotherapy. Oxidative stress and inflammation are the major pathomechanisms of Mtx-induced nephrotoxicity. Alamandine (Ala), a new member of the renin-angiotensin system (RAS), is an important peptide with antioxidant and anti-inflammatory capacities. In this study, it was investigated whether Ala ameliorates Mtx-induced kidney damage by reducing oxidative stress and inflammation.

**Materials and Methods:** Male Wistar albino rats were assigned into three groups: control group, Mtx group, and Mtx+Ala group. At the end of the experiment, kidney tissues were quickly removed. Glutathione (GSH) and malondialdehyde (MDA) levels were measured to determine the oxidative state in kidney tissues. In addition, tissue samples were assessed as histopathological and immunohistochemical for heat shock protein 60 (HSP60), caspase-3, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and receptor-interacting protein kinase-3 (RIPK3).

**Results:** Mtx treatment resulted in reduced GSH content, elevated MDA level, increased heat shock protein 60 (HSP60), and caspase-3 expression. These changes in kidney tissues of rats treated with Mtx triggered oxidative stress characterized by apoptosis and kidney damage. Mtx also markedly increased the expression of TNF- $\alpha$ , an inflammation marker, and RIPK3, a marker of necroptosis. However, Ala administration significantly alleviated Mtx-induced kidney damage by reducing apoptosis and necroptosis by suppressing oxidative stress and inflammation.

**Conclusion:** Taken together, our results support that Ala treatment can serve as a new and promising therapeutic strategy against Mtx-induced nephrotoxicity.



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## Introduction

Chemotherapy, which continues to play a vital role in the treatment of cancer, is widely preferred because surgery and radiotherapy may not be completely effective in advanced stages of malignancy. However, a lot of chemotherapeutic agents cause side effects that are considered the main cause of morbidity and require hospitalization in intensive care units [1].

Methotrexate is a powerful chemotherapeutic agent widely used in the treatment of various malignancies. Also, low doses of Mtx are used in the treatment of non-malignant conditions such as autoimmune and inflammatory disorders such as rheumatoid arthritis and psoriasis [2, 3]. On

the other hand, nephrotoxicity is one of the major problems caused by Mtx therapy, complicating the treatment regimen and limiting its effectiveness. High-dose Mtx treatment may create acute renal failure by causing a condition termed crystalline nephropathy due to Mtx sedimentation in kidney tubules. Constant treatment with low concentrations of Mtx may also result in serious toxicities [4]. It has been proven that nephrotoxicity due to Mtx therapy is based on multiple damage mechanisms. First of all, it has been reported that the cytotoxic effects of Mtx are not only against tumor cells but also against normal cells. In addition, Mtx-induced nephrotoxicity has been reported to be associated with elevated oxidative stress and elevated levels of proinflammatory cytokines that can induce cell death. It has also been noted that Mtx causes cells to become vulnerable to oxidative stress by disrupting

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the antioxidant defense system [5].

The search for nephroprotective agents to reduce Mtx-induced toxicity and enlarge Mtx's therapeutic potency is very important for the safe use of this multi-targeted drug. Therefore, interest in Ala, one of the peptides involved in the protective pathways of the RAS, has increased. Ala, identified as a novel peptide of RAS is a vasoactive peptide claimed to have protective effects like Ang (1-7) [6]. It is produced by decarboxylation of angiotensin (Ang)-(1-7) or hydrolysis of Ang A. A selective receptor, mas-related G-linked protein receptor member D, has been reported to mediate the actions of Ala [7, 8]. The activities of Ala have mainly been studied in the cardiovascular context. It has been shown that Ala attenuates inflammation, apoptosis, and autophagy in sepsis-induced myocardial injury caused by lipopolysaccharide [9]. In addition, Ala administration has been shown to alleviate cardiac dysfunction and reduce cardiac fibrosis through the inhibition of oxidative stress [10].

A few studies have reported the protective role of Ala on renal hemodynamics and functions. Moreover, it has been noted that Ala is not only a physiological regulator of renal homeostasis but also has a significant role in the pathophysiology of kidney diseases. The outputs of these studies emphasized that Ala ameliorates nephrotoxicity by reducing oxidative stress and apoptosis, followed by inflammation [11-13]. There are limited studies investigating the effects of Ala against nephrotoxicity. For this purpose, in the current study, it was purposed to investigate biochemical, histopathological, and immunohistopathological paths of whether Ala can alleviate inflammation, apoptosis, and necroptosis by suppressing Mtx-induced oxidative stress in kidney tissue.

## Materials and Methods

### *Animals and experimental procedure*

Thirty male Wistar albino rats aged 4-6 months were used in our study. All processes performed on animals were based on the National Institutes of Health Animal Research Guidelines and ARRIVE guidelines [14] with the confirmation of the Inonu University Faculty of Medicine Animal Research Ethics Committee (Protocol: 2021/22-10). The animals housed under the controlled standard conditions (rf) were randomly divided into three equal groups (ten in each group) as follows after acclimatization for one week. A software program developed by Arslan et al. [15] determined the number of rats in each group.

The control group only received the saline daily by intraperitoneal administration (i.p.) throughout the experiment. The Mtx group received a single dose of Mtx (20 mg/kg, Koçak, Istanbul, Turkey) by i.p. on the 6<sup>th</sup> day of the study [16]. The Mtx+Ala group received a single dose of Mtx (20 mg/kg) by i.p. on the 1<sup>st</sup> day and the ALA (50 µg/kg/day) daily by i.p. between the 6<sup>th</sup>-10<sup>th</sup> days of the experiment [17].

The experiment was terminated on the 11<sup>th</sup> day. The kidneys of the rats under ketamine/ xylazine anesthesia were quickly removed. The right kidney was fixed in 10% formaldehyde for further histological analysis, and the left kidney was preserved at -80 °C to determine oxidative stress parameters including MDA and GSH.

### *Biochemical analysis*

To calculate the levels of MDA and GSH, protein concentration in the renal tissue was determined as described by the Biuret method [18] and a calibration curve was developed using bovine serum albumin.

The determination of the MDA level was carried out as described by Mihara and Uchiyama [19]. 10% homogenate was obtained from tissue samples homogenized in 1.15% potassium chloride solution. The tissue homogenates were blended with 0.6% thiobarbituric acid and 1% phosphoric acid solutions and the tubes were placed in a boiling water bath for 45 min. Then, 2 ml of n-butanol was added to the tubes and the tubes were vortexed for 5 min and centrifuged for 10 minutes. In this way, the supernatant was obtained from tissue samples. The absorbance of the supernatants was measured at 532 nm. A standard curve was prepared from a standard solution of 1,1,3,3-tetramethoxypropane, and results were given in nmol per g tissue (nmol/g tissue).

Ellman's method was used to determine the GSH level [20]. 10% homogenate was formed by homogenizing tissue samples. Afterward, the homogenates were centrifuged to obtain the supernatant. The trichloroacetic acid solution was mixed with the supernatant, and the resulting solutions were centrifuged again to make the sample ready for the determination of GSH. The final solutions were vortexed, and the formation of 5-thio-2-nitrobenzoate was followed spectrophotometrically at 412 nm for 5 min. The obtained results were evaluated from the GSH standard graph and were given in nmol per g tissue (nmol/g tissue).

### *Histological analysis*

After fixation with formaldehyde, tissue samples were performed dehydration and clearing processes, and embedded in paraffin. Subsequently, 4 µm sections of the paraffin blocks were taken by microtome, then the sections were mounted onto microscopical slides and were stained with hematoxylin&eosin (H&E) for general histological evaluation, were stained periodic acid Schiff (PAS) for brush border determination. All slides were scored in terms of tubular dilatation and swelling, necrosis, desquamation, and loss of brush border of tubule cells. Ten randomly selected areas were examined in each slide and were semi-quantitatively assigned as follows according to the severity of the above histopathological alterations: 0: no change, 1: mild, 2: moderate, 3: severe change [21].

### *Immunohistochemical analysis*

Immunohistochemical staining was carried out to demonstrate HSP60 as a mitochondrial stress marker, TNF-α as an inflammation marker, caspase-3 as an apoptosis marker, and RIPK3 as a necroptosis marker (Santa Cruz Biotechnology, Inc., Heidelberg, Germany, HSP60: 1:100 dilution, sc-13115; TNF-α: 1:100 dilution, sc-52746; caspase-3: 1:100 dilution, sc-56053; and RIPK3: 1:100 dilution, sc-374639).

After deparaffinization and rehydration, tissue sections were boiled in a citrate buffer solution for 20 minutes for antigen retrieval. Then, the tissue specimens were treated

with 3% hydrogen peroxide for 12 min to block endogenous peroxidase activity. The protein block was applied to the sections for 5 min to block non-specific binding. Afterward, the tissue sections were incubated for 60 min with primary antibodies (HSP60, TNF- $\alpha$ , caspase-3, and RIPK3). After protein blockage, the slides were incubated for 20 min with a biotinylated-secondary antibody and then, were treated for 20 min with streptavidin-biotin peroxidase. The immunological reaction was visualized with aminoethyl carbazole chromogen. The slides stained with hematoxylin were rinsed in tap water, and coverslipped.

For immunohistochemical evaluation, ten randomly selected areas in each slide were examined and the immunostaining was semi-quantitatively scored based on the prevalence of immunoreactivity (0: 0-25%, 1:26-50, 2:51-75%, 3:76-100%) and the intensity of immunoreactivity (0: none, 1: mild, 2: moderate, 3: severe). The total score was calculated as follows [22].

The total score = (immunostaining prevalence) X (immunostaining intensity)

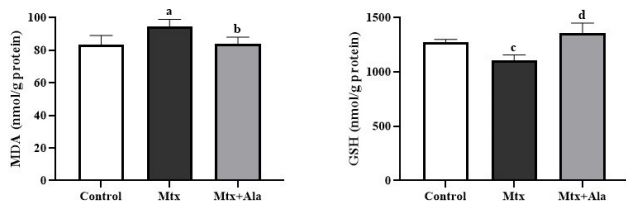
### Statistical analysis

The IBM SPSS 26 software program for Windows was used for the statistical analysis of the data (SPSS Inc., Chicago, IL). The Shapiro-Wilk test was used to understand whether the data were normal distribution. Normally distributed data were analyzed by one-way ANOVA and following the Tukey post hoc analysis was used to multiple comparisons between the groups. Non-normal distributed data were analyzed by the Kruskal-Wallis, and pairwise comparisons were performed using the Bonferoni corrected Mann-Whitney U test. A value of  $p < 0.05$  was taken into account as statistically significant. Data were expressed as median (minimum-maximum) or mean  $\pm$  standard deviation depending on the overall variant distribution.

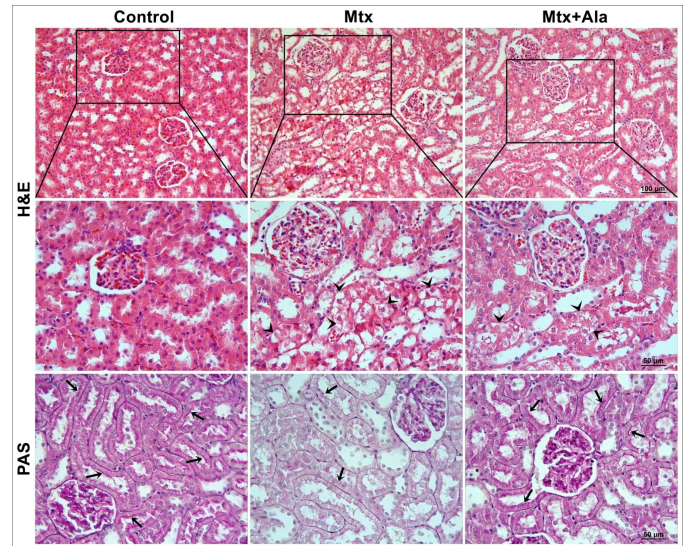
## Results

### Biochemical results

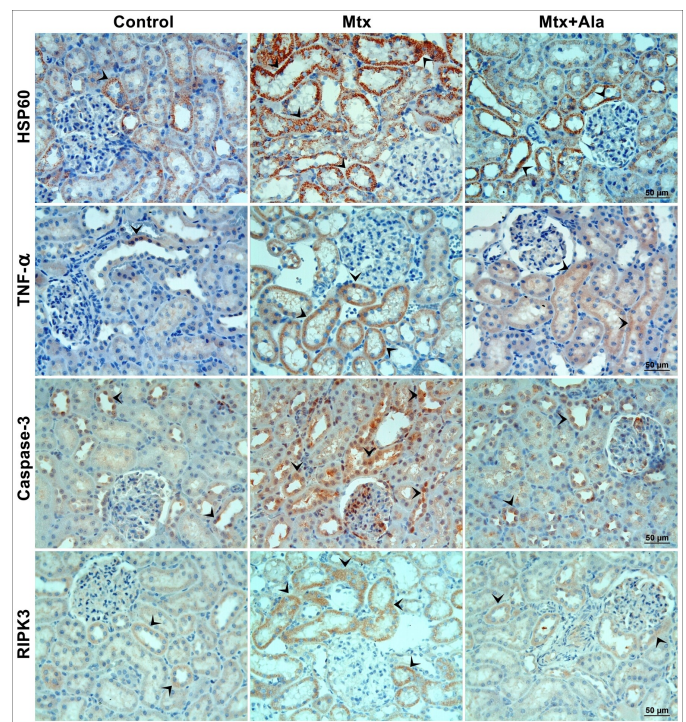
For the control, Mtx, and Mtx+Ala groups, tissue MDA levels were measured as  $83.2 \pm 5.8$  nmol/g,  $94.6 \pm 4.3$  nmol/g, and  $83.8 \pm 4.2$  nmol/g respectively. The Mtx



**Figure 1.** Distribution of tissue levels of MDA and GSH according to the groups. Data are expressed as mean  $\pm$  standard deviation. <sup>a</sup>Increase compared with the control group ( $p < 0.05$ ). <sup>b</sup>Decrease compared with the Mtx group ( $p < 0.05$ ). <sup>c</sup>Decrease compared with the control group ( $p < 0.05$ ). <sup>d</sup>Increase compared with the Mtx group ( $p < 0.05$ ).



**Figure 2.** Photomicrographs showing effects of Ala on histopathological changes in Mtx-induced nephrotoxicity. Arrowheads and arrows indicate necrotic cells and brush borders, respectively.



**Figure 3.** Photomicrographs showing the effect of Ala on HSP60, TNF- $\alpha$ , caspase-3, and RIPK3 immunoreactivity on MTX-induced nephrotoxicity. Arrowheads indicate immunoreactive cells.

group's MDA level was significantly higher than the control group ( $p < 0.05$ ). The Mtx+Ala group's MDA level was prominently lower than the MTX group ( $p < 0.05$ ) (Figure 1).

For the control, Mtx, and Mtx+Ala groups, tissue GSH levels were measured as  $1272 \pm 26.6$  nmol/g,  $1105 \pm 51.1$  nmol/g, and  $1357.8 \pm 90.6$  nmol/g respectively. The Mtx

**Table 1.** The results of the histopathological evaluation of each group.

Groups	Tubular degeneration	Loss of brush border
Control	0 (0 - 1)	0 (0 - 1)
Mtx	1 (0 - 3) <sup>a</sup>	1 (0 - 2) <sup>a</sup>
Mtx+Ala	0 (0 - 2) <sup>b</sup>	0 (0 - 1) <sup>b</sup>

Data are given as median (minimum-maximum). <sup>a</sup>Increase compared to the control group ( $p < 0.0001$ ). <sup>b</sup>Decrease compared to the MTX group ( $p < 0.0001$ ).

**Table 2.** The results of the immunohistochemical evaluation of each group.

Groups	HSP60	TNF- $\alpha$	Caspase-3	RIPK3
Control	3 (1 - 6)	4 (0 - 8)	4 (1 - 6)	2 (0 - 4)
Mtx	4 (2 - 9) <sup>a</sup>	4 (3 - 12) <sup>a</sup>	4 (2 - 9) <sup>a</sup>	4 (0 - 9) <sup>a</sup>
Mtx+Ala	3 (1 - 9) <sup>b</sup>	4 (0 - 9) <sup>b</sup>	4 (2 - 6) <sup>b</sup>	2 (0 - 6) <sup>b</sup>

Data are given as median (minimum-maximum). <sup>a</sup>Increase compared to the control group ( $p < 0.001$ ). <sup>b</sup>Decrease compared to the MTX group ( $p < 0.01$ ).

group's GSH level was significantly lower than the control group ( $p < 0.05$ ). The Mtx+Ala group's GSH level was prominently higher than the Mtx group ( $p < 0.05$ ) (Figure 1).

#### Histopathological results

The control group exhibited a normal histological structure of renal tissue except for slight tubular epithelial desquamation in slides stained with H&E. The brush border of proximal tubules in this group was observed as intact in the PAS-stained slides (Figure 2). However, remarkable tubular damage was observed in the Mtx group. In tissue slides stained with H&E belonging to this group, marked necrotic changes, epithelial desquamation, and dilatation were observed in the renal tubules. In addition, loss of brush border in proximal tubule epithelium was remarked in PAS-stained slides in the Mtx-treated group (Figure 2). The difference between the control group and the Mtx group in terms of histopathological changes was found to be statistically significant. On the other hand, Ala significantly improved Mtx-induced renal tubular damage (Figure 2). The improvement observed in the renal tubules in the Mtx+Ala group was statistically significant when compared with the Mtx group ( $p < 0.05$ ). The results of the histopathological evaluation of the renal tissue of each group are expressed in Table 1.

#### Immunohistochemical results

The immunoreactivity was observed as brown staining in the cytoplasm of renal tubule cells (Figure 3). The slight immunoreactivity of HSP60, TNF- $\alpha$ , RIPK3, and caspase-3 was seen in the renal tubules of the control group. Compared with the control group, the immunoreactivity of HSP60, TNF- $\alpha$ , RIPK3, and caspase-3 was markedly increased in the Mtx-treated group's renal tubules ( $p < 0.05$ ).

In the Mtx+Ala group, the severity of all the proteins' immunoreactivity was significantly reduced compared to the Mtx group ( $p < 0.01$ ). The results of the evaluation for HSP60, TNF- $\alpha$ , caspase-3, and RIPK3 proteins for each group are expressed in Table 2.

#### Discussion

The current results showed that Ala has therapeutic effects that reduce Mtx-induced biochemical, immunohistochemical, and histological alterations and restore injured kidney tissues to their almost normal structures.

Mtx is widely utilized in the treatment of varied illness, especially cancer. Although Mtx has beneficial effects in treatment, its use is limited due to its acute toxic effects on vital organs such as kidney, liver, testis, and heart [1, 23]. One of the main problems associated with the use of Mtx is nephrotoxicity. Mtx-induced nephrotoxicity may develop due to multiple damage mechanisms and one of the main mechanisms is oxidative stress [4, 5]. Oxidative stress is an unbalance between the formation of reactive metabolites such as free radicals and their elimination by protective mechanisms called antioxidants or radical scavengers. This unbalance induces metabolic alterations that caused necroptosis and apoptosis by leading to the destruction of cellular molecules such as DNA, proteins, and lipids [24, 25].

In this study, the effects of Mtx treatment on oxidative stress were biochemically evaluated by measuring MDA and GSH levels. MDA, an end-metabolite generated by the lipid peroxidation cascade, is used as one of the popular biomarkers of oxidative stress. GSH is a substantial component of the cell's protective mechanism against oxidative stress, so its decrease is often considered an indicator of oxidative stress [25]. Our data revealed a prominent decrease in GSH and a significant increase in MDA in the kidney of Mtx-treated rats. These results were consistent with previous studies documenting decreased GSH and increased MDA following Mtx administration [26, 27]. In this case, the formation of oxidative attacks may cause macromolecule damage and in this way cause harmful effects on all components of the cell. Indeed, in this study, it was observed that Mtx caused histological damage such as necrosis, desquamation, and loss of brush border in renal tubule cells. It has been documented in previous studies that Mtx treatment causes such histological changes in the kidney [2, 5].

In the current study, the fact that Mtx causes oxidative stress and cellular destruction was also supported by evaluating the expression level of HSP60. The results of our immunohistochemical analysis showed that Mtx treatment caused a prominent increase in HSP60 expression in renal tissues. HSPs, which are highly sensitive chaperones, are expressed at a low level under normal conditions, but HSP expression is quickly increased in cells exposed to stress for a variety of reasons, such as starvation, elevated temperature, UV, chemicals, or pharmacological agents. Outside their association with cellular resuscitation, they are also related to cellular damage and excessively expressed against oxidative stress. HSP60, which has predominantly a role in the folding of proteins in the mitochondrial matrix, is localized in mitochondria and it has been reported

that mitochondria release HSP60 into the cytosol under oxidative stress [28, 29].

In this study, it was revealed that the decrease in GSH contents and increase in MDA levels induced by Mtx in the kidney were alleviated by Ala treatment. In addition, Ala treatment also decreased HSP60 expression, which was increased due to Mtx administration. These results suggest that Ala ameliorates cells and tissues against Mtx-induced oxidative stress. In the current study, Ala provided significant histological improvement in tissue in parallel with the reduction of oxidative stress caused by Mtx. Consistent with our results, many studies have shown that Ala exhibits antioxidant properties and reduces oxidative damage in tissues caused by various harmful factors [11–13].

Increasing evidence has shown that Mtx treatment triggers apoptosis by inducing oxidative stress [5, 30]. Apoptosis occurs by plural cellular signaling cascades resulting in the activation of caspases or proteases. Caspase-3, one of the significant apoptotic enzymes, is a sign of the irreversible point of apoptosis [31]. Previous studies have demonstrated the overexpression of caspase-3 in Mtx treatment [30, 32]. Our immunohistochemical analysis showed strong caspase-3 expression in kidney tissue in the Mtx-treated group. On the other hand, administration of Ala markedly inhibited caspase-3 expression. The anti-apoptotic effect of Ala on kidney tubule cells can be explained by its role in reducing oxidative stress, as previously reported [11].

In the present study, Mtx treatment led to a prominent increase in RIPK3 expression. RIPK3 is an important protein in the regulated cell death pathway that mimics features of apoptosis and necrosis called necroptosis [33]. A previous study has shown that necroptosis is involved in Mtx-induced tissue toxicity [34]. Increasing evidence has indicated that inflammation causes necroptosis in renal tissues. Inflammatory reactions are activated due to increased oxidative stress during Mtx treatment, resulting in the formation of pro-inflammatory proteins including TNF- $\alpha$ , interleukin-6 (IL-6), and interleukin-1 $\beta$  (IL-1 $\beta$ ) [5, 27]. TNF- $\alpha$  is a potent proinflammatory protein and high TNF- $\alpha$  levels may trigger necroptosis signals that caused cell/tissue injury [34]. In the present study, MTX treatment also caused increased expression of RIPK3 as well as increased expression of TNF- $\alpha$  in kidney tissues. RIPK3 is a particularly important determinant in TNF- $\alpha$ -induced necrotic cell death and mediates necroptosis in the presence of elevated levels of TNF- $\alpha$  [35]. Necroptosis exhibits the main morphological properties of necrosis, including swelling of the cell organelles, plasma membrane rupture, and leakage of intracellular components due to the eventual lysis of the cell [36]. Consistent with the outputs of our study, it was histologically observed that Mtx treatment caused necrotic changes in the renal tubule.

On the contrary, in this study, the administration of Ala decreased the level of increased expression of TNF- $\alpha$  and RIPK3 caused by Mtx. Previous studies have shown that Ala inhibits nuclear factor kappa B (NF- $\kappa$ B) activity in various inflammatory conditions [13, 37]. NF- $\kappa$ B activation, which has a crucial role in the pathogenesis of kidney inflammation due to MTX therapy, regulates multiple genes associated with kidney diseases and induces inflammatory reactions by mediating the TNF- $\alpha$ , IL-6, and IL-1 $\beta$

expression. Therefore, the inactivation of NF- $\kappa$ B signaling pathways is accompanied by a decrease in inflammatory cytokine levels in kidney tissues [38, 39]. In this study, reduced expression of TNF- $\alpha$  and RIPK3 was accompanied by marked histologically attenuated necrotic changes in renal tissues. Consistent with other findings reported in the literature, the result of this study showed that Ala treatment ameliorated the kidneys against Mtx by reducing proinflammatory factors.

## Conclusion

Current findings have shown that Ala has antioxidant effects by modulating the oxidative state caused by Mtx therapy in the kidney tissue. Moreover, the current study demonstrated that Ala against the toxic effects of Mtx has anti-inflammatory, anti-apoptotic, and anti-necrotic effects using biochemical, histological, and immunohistochemical parameters. In line with the data obtained from our study and the knowledge in the literature, Ala has a therapeutic role in cellular protection and repair processes against Mtx-induced nephrotoxicity. On the other hand, this study's limitation is that Ala's therapeutic effect on nephrotoxicity is not well explored through cellular mechanisms. Therefore, further investigations are necessary for the potential use of Ala in nephrotoxicity that may develop due to chemotherapy where Mtx is used as a first-line drug.

## Authors contributions

All authors participated in the conception and design of the study. M.Y.A., M.G., and S.P. carried out the experimental procedures. A.Y. and N.V. conducted histochemical and immunohistochemical experiments, analysis, and interpretation. Y.C. and M.D. conducted biochemical experiments, analysis, and interpretation. The manuscript was composed by A.Y. and all authors perused and confirmed the final manuscript.

## Ethical approval

This study was carried out with the approval of the Ethical Committee of Experimental Animals of the Faculty of Medicine at Inonu University (2021/22-10).

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## Declaration of competing interest

The authors have no conflict of interest to declare.

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