

Alpha-Pinene Attenuates Methotrexate-Induced Kidney Damage

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This investigation assessed the potential of alpha-pinene, a natural compound that can counteract inflammation, oxidative stress, cell death and tumorigenesis, could prevent methotrexate-induced renal injury, a clinically significant complication of this frequently used chemotherapeutic agent. Sprague-Dawley rats were divided into five groups: control (CTR, no treatment), vehicle (VEH; dimethyl sulfoxide (DMSO) injected for fourteen days), alpha-pinene (AP, 50 mg/kg, daily for fourteen days), methotrexate (MTX, 20 mg/kg on 12th day of DMSO administration), and combined alpha-pinene and methotrexate treatment (APM). In the MTX group, considerable increases in blood urea nitrogen (BUN), serum creatinine, cystatin C, urinary neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (Kim-1), as well as tissue levels of malondialdehyde (MDA) and Kim-1, were observed, along with a marked decrease in glomerular filtration rate (GFR). In the APM group, BUN, creatinine, cystatin C, NGAL, and GFR values showed partial improvement, while the reductions in Kim-1 and MDA levels were more pronounced. Histopathological inspection demonstrated that alpha-pinene pretreatment considerably minimized methotrexate-induced tubular and glomerular deterioration. Our findings indicate that, although alpha-pinene does not completely prevent methotrexate-induced renal injury, it shows strong potential as a protective and supportive therapeutic agent against nephrotoxicity. More research is needed to reveal its molecular mechanisms of action and determine the optimal dosage for maximizing its renoprotective efficacy.

Keywords: Alpha-pinene, Nephrotoxicity, NGAL, Kim-1, Cystatin C, GFR

INTRODUCTION

Alongside the rising incidence of cancer worldwide, the demand for chemotherapeutic agents has also increased significantly. Methotrexate, a folic acid antagonist, is extensively used to fight hematological malignancies, solid tumors and inflammatory states, such as rheumatoid arthritis and psoriasis. However, despite its therapeutic efficacy, methotrexate has been documented to cause serious detrimental effects on internal organs, most notably the kidneys, liver, reproductive organs and gastrointestinal system¹⁻³. Since this chemotherapeutic agent is primarily eliminated by the kidneys, this organ is particularly susceptible to methotrexate related toxicity. Methotrexate not only causes mechanical damage through crystallization within the renal tubules but also induces noticeable renal dysfunction by initiating

several pathophysiological mechanisms, including oxidative stress, inflammation, and apoptosis⁴⁻⁸.

Currently, extensive research efforts are focused on developing strategies that prevent or alleviate the harmful actions of methotrexate while maintaining its therapeutic efficacy. One promising approach is to use natural compounds as adjuvant agents⁴⁻⁸. In particular, alpha-pinene, a coniferous monoterpene, has attracted considerable interest. Owing to its pleasant aroma and high volatility, alpha-pinene is widely utilized in the fragrance, food, cleaning, and personal care industries⁹. Subsequent in vitro and in vivo pharmacological studies have revealed the remarkable therapeutic potential of alpha-pinene, which originates from its ability to reduce oxidative and inflammatory injury as well as programmed cell death¹⁰.

The antioxidant action of alpha-pinene is mainly depends on its capability to induce the production of endogenous defence enzymes including catalase,

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glutathione peroxidase and superoxide dismutase by activating the nuclear factor erythroid 2-related factor 2 (Nrf2) antioxidant signalling pathway. This activation leads to a reduction in malondialdehyde (MDA) levels -an indicator of lipid peroxidation- and an elevation in cellular glutathione content¹¹⁻¹⁴. The anti-inflammatory characteristics of alpha-pinene are associated with the blunting some signalling pathways including mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF- κ B). This leads to an important reduction in the production of key cytokines of inflammation, namely interleukin-1 beta (IL-1 β), tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6)¹⁵⁻¹⁷. The anti-apoptotic impact of alpha-pinene is linked to a reduction in the Bcl-2-associated X protein (Bax)/B-cell lymphoma 2 (Bcl-2) ratio and the suppression of the executive caspase activation^{18, 19}. On the other hand, it has also been reported that this monoterpene induces apoptosis and exhibits anti-proliferative properties in tumour cells. This is achieved through the induction of cell cycle arrest, the promotion of caspase-3 activation, the triggering of mitochondrial damage, the increase of oxidative stress, the suppression of survival signalling pathways, and the enhancement of natural killer cell cytotoxicity²⁰⁻²³.

Previous studies have demonstrated that alpha-pinene has remarkable protective effects, modulating key cellular mechanisms such as oxidative stress, endoplasmic reticulum stress, neuroinflammation and apoptotic cell death in diverse experimental injury models^{18, 24-27}. In line with these findings, our previous studies have demonstrated the testiculoprotective, cardioprotective, and hepatoprotective properties of alpha-pinene during the methotrexate toxicity^{12, 28}. Although the biological activities of alpha-pinene have been reported in various experimental models, its precise role in methotrexate-induced renal injury has not yet been elucidated. Therefore, the current study aimed to investigate the protective effects of alpha-pinene against methotrexate-induced renal damage and functional impairment using both conventional biochemical parameters and sensitive renal biomarkers, together with histopathological evaluation. We hypothesized that alpha-pinene would attenuate methotrexate-induced nephrotoxicity by reducing oxidative tissue damage and preserving renal histological integrity. To the best of our knowledge, this is the first study evaluating the renoprotective effects of alpha-pinene in methotrexate-induced nephrotoxicity model.

MATERIALS AND METHODS

Experimental design and grouping

A total of 37 male Sprague-Dawley rats (2.5-3-months old) were used in this experimental study and were randomly divided into five groups. The Control (CTR, n=7) group underwent no treatment. The Vehicle (VEH, n=7) group was given subcutaneous (s.c.) injections of 0.1% dimethyl sulfoxide (DMSO), which was used to dissolve alpha-pinene, for fourteen days. The MTX group (n=8) received DMSO in the same manner and was also administered methotrexate (20 mg/kg, s.c.) on the 12th day of DMSO administration. The AP group (n=7) received alpha-pinene treatment once a day (50 mg/kg, s.c.) for fourteen days. The combined methotrexate and alpha-pinene treatment (APM) group (n=8) received alpha-pinene for fourteen days, with one dose of methotrexate on 12th day²⁸.

The day before they were sacrificed, the animals were individually placed in metabolic cages where their daily food and water intake was noted, and urine was sampled. After fourteen days, all the animals were anesthetized with ketamine (80 mg/kg, s.c.) and xylazine (10 mg/kg, s.c.), then sacrificed. Blood samples and kidney tissues were collected and used to measure the parameters listed below. The Local Animal Ethics Committee approved all the procedures for this study, which were subsequently carried out in accordance with national and international guidelines on the care and use of laboratory animals (Decision no. 2023-5/21, 6 November 2023).

Biochemical parameters

Creatinine levels in serum and 24-hour urine samples were measured using a spectrophotometric method with commercial kits (Otto BC139, Otto Scientific, Ankara, Turkey) on an autoanalyzer (Mindray BS-400, Shenzhen, China). The obtained data and the urine flow rate were used to estimate the glomerular filtration rate (GFR). Serum urea levels were determined using a kinetic method with appropriate commercial kits (Otto BC157, Otto Scientific, Ankara, Turkey) on the same device, and blood urea nitrogen (BUN) values were calculated from the results.

Serum cystatin C, urinary neutrophil gelatinase-associated lipocalin (NGAL), and kidney injury molecule-1 (Kim-1) concentrations in urine and left renal tissue were quantified using commercially supplied enzyme-linked immunosorbent assay (ELISA) kits (Bioassay Technology Laboratory,

E0145Ra, E0762Ra and E0549Ra, respectively). All procedures, including sample preparation, were executed in accordance with the manufacturer's specifications. Standards supplied with the kits were analysed alongside the samples. The concentrations of the target proteins were determined by generating standard curves, and are expressed in ng/mL, except for tissue Kim-1 levels, which were adjusted for overall protein concentration and are presented in ng/mg protein.

The spectrophotometric determination of MDA levels in left kidney tissue homogenates was performed by monitoring, at 535 nm, the chromogenic complex produced by the reaction of MDA with thiobarbituric acid under acidic conditions²⁹. As the MDA standard, 1,1,3,3-tetramethoxypropane was used and, MDA concentrations in the tissue homogenates were calculated from the corresponding standard curve. The results were standardized to the total protein concentration of individual samples and reported as nmol/mg protein.

Histological evaluation

The right kidneys of the animals were fixed in 10% neutral formalin. Following routine histological procedures specific to kidney tissue, the specimens were embedded in paraffin wax and then blocks were cut into sections (5 µm-thick) using a microtome. The overall histological architecture of the kidneys was revealed and the impact of methotrexate and alpha-pinene at the tissue level was investigated by staining the sections with haematoxylin and eosin (H&E). At least 5 different areas in each section were examined by 2 histologists, blinded to the treatment groups, at different times under a light microscope, scored for tissue damage and cellular degeneration, and photographed. The scoring evaluation was performed on a scale of 0 to 3: 0 = no structural change; 1 = slight structural change; 2 = moderate structural change; 3 = significant structural change⁶⁻⁸.

Statistical analysis

The results were expressed as mean ± standard deviation. Normality of the data was analysed using the Kolmogorov-Smirnov test. Normally distributed data were analysed by one-way analysis of variance (ANOVA), subsequently followed by Tukey's post hoc test. Histopathological scores, which did not follow a normal distribution, were analysed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test. A p value of less than 0.05 was regarded statistically significant. GraphPad Prism software (4.0) was used to perform data analyses.

RESULTS

Changes in body weight and feeding behaviour

There were no obvious disparities between the groups in relation to initial body weight, final body weight or relative kidney weight. Animals in the CTR, VEH and AP groups exhibited similar weight gains. However, weight gain in the MTX and APM groups was lower than in the other groups (see Table 1). Metabolic cage measurements revealed no significant changes in daily water intake across the groups. Nevertheless, food consumption was considerably reduced in the MTX and APM groups ($p < 0.05$) relative to the CTR group (Table 1).

Biochemical variables

Serum creatinine levels

Serum creatinine concentrations in control animals (0.573 ± 0.057 mg/dL) remained unaffected by DMSO or alpha-pinene administration but increased significantly following methotrexate injection (0.801 ± 0.072 mg/dL). Serum creatinine concentrations in the APM group (0.678 ± 0.060 mg/dL) were considerably lower than in the MTX group ($p < 0.05$), but higher than in the CTR group ($p < 0.05$; see Figure 1A).

GFR

The GFR values of the CTR, VEH and AP groups were similar. A substantial decline in GFR was

Table 1 — Changes in Body Weight and Consumption Parameters

Groups	Body weight change (%)	Relative kidney weight (%)	Food intake (g)	Water intake (mL)
CTR	8.18±0.97	0.852±0.07	21.6±6.5	26.43±7.48
VEH	7.43±5.25	0.852±0.04	16.8±6.0	22.14±6.99
AP	8.55±6.25	0.889±0.09	20.5±3.3	25.71±4.50
MTX	0.56±5.95* ϕ	0.807±0.06	10.1±9.3 *	23.75±7.44
APM	-1.59±4.12** ϕ ϕ	0.831±0.04	10.6±8.4 *	20.00±5.77

Statistical differences from the CTR group are indicated as * $p < 0.05$, ** $p < 0.01$; statistical differences from the VEH group are indicated as # $p < 0.05$; statistical differences from the AP group are indicated as ϕ $p < 0.05$, $\phi\phi$ $p < 0.01$.

calculated in the MTX group (0.383 ± 0.098 mL/min). The GFR value of the APM group (0.540 ± 0.080 mL/min) was lower than those of the CTR and VEH groups but remained higher than that of the MTX group ($p < 0.05$; see Figure 1B).

BUN levels

Neither the DMSO nor the alpha-pinene treatment caused any significant change in BUN levels. However, methotrexate treatment significantly increased BUN levels to 44.33 ± 6.15 mg/dL ($p < 0.001$). In the APM group that received alpha-pinene pretreatment, this value was significantly lower at 29.52 ± 9.72 mg/dL ($p < 0.05$) than in the MTX group ($p < 0.05$), but significantly higher in the VEH group ($p < 0.05$) group (Figure 1C).

Serum cystatin C levels

Serum cystatin C levels were similar across the CTR (5.68 ± 1.37 ng/mL), VEH (6.53 ± 1.96 ng/mL) and AP (6.14 ± 1.74 ng/mL) groups. However, this value was considerably greater in the MTX group (11.43 ± 2.09 ng/mL). In the APM group, serum cystatin C concentration was lower than in the MTX group ($p < 0.05$), but higher than in the CTR group ($p < 0.05$; see Figure 1D).

Urinary NGAL levels

Urinary NGAL levels were measured as 8.10 ± 1.58 ng/mL in the CTR group. This value was close to CTR in the VEH and AP groups and was considerably higher in the MTX group (16.78 ± 2.39 ng/mL). The urinary NGAL concentration in the APM group was 11.80 ± 2.33 ng/mL, which was below the level seen in the MTX group ($p < 0.01$) and above the level in the CTR group ($p < 0.05$; Figure 2A).

Urinary Kim-1 levels

The amounts of Kim-1 in the urine samples were similar among the CTR (0.603 ± 0.262 ng/mL), VEH (0.572 ± 0.148 ng/mL) and AP (0.639 ± 0.188 ng/mL) groups. However, the MTX group had a higher level (1.212 ± 0.149 ng/mL) than the other groups. In the APM group, the level was 0.863 ± 0.168 ng/mL, which was clearly less than in the MTX group ($p < 0.01$; see Figure 2B).

Renal Kim-1 levels

Kim-1 levels were similar in the CTR (0.116 ± 0.014 ng/mg protein), VEH (0.133 ± 0.033 ng/mg protein) and AP (0.124 ± 0.030 ng/mg protein) groups. Methotrexate administration markedly elevated Kim-1 to 0.203 ± 0.027 ng/mg protein level ($p < 0.001$). In the APM group, Kim-1 was measured

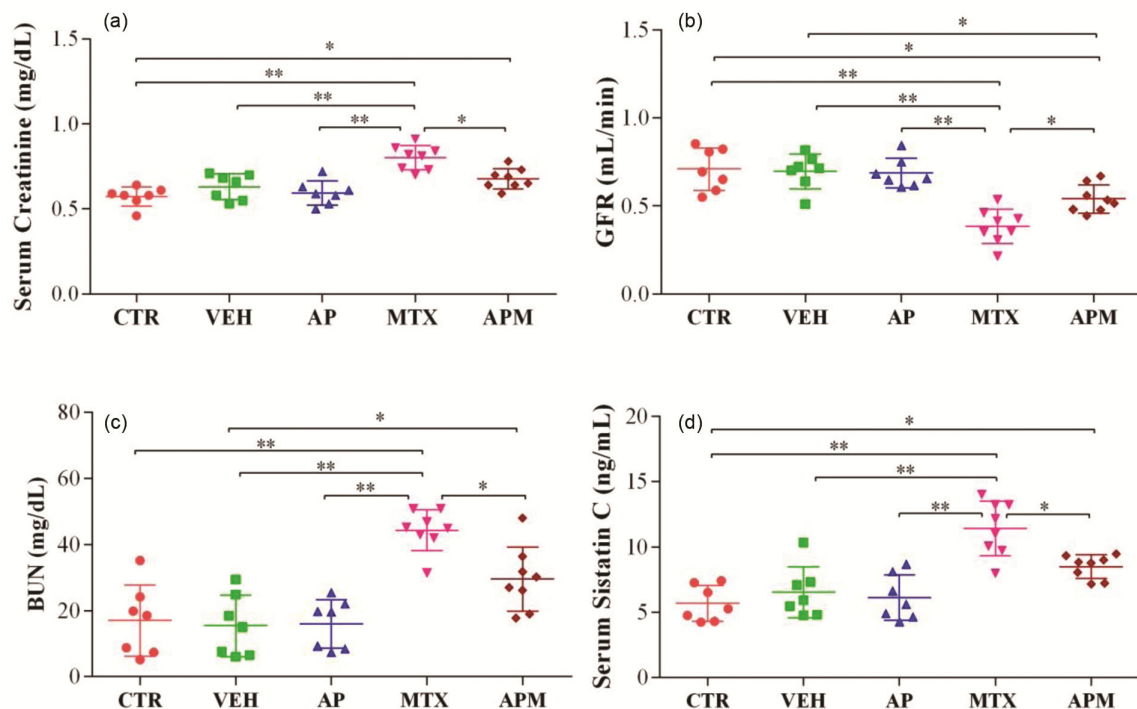


Figure 1 — Serum creatinine (A), GFR (B), BUN (C) and serum cystatin C (D) levels in all groups. Intergroup statistical differences were indicated as * $p < 0.05$, ** $p < 0.001$.

at 0.148 ± 0.020 ng/mg protein, representing a significant reduction with respect to the MTX group ($p < 0.01$), with no substantial change compared to the CTR group (see Figure 2C).

Renal MDA levels

MDA levels were comparable among the CTR, VEH and AP groups (0.145 ± 0.034 ; 0.142 ± 0.022 and 0.121 ± 0.056 nmol/mg protein, respectively) and increased to 0.290 ± 0.059 nmol/mg protein levels in the MTX group ($p < 0.001$, vs. CTR, VEH and AP). In the APM group, it was measured at 0.204 ± 0.020 nmol/mg protein, which was significantly below the level in the MTX group ($p < 0.01$), but above that in the AP group ($p < 0.01$; see Figure 3).

Histological Evaluation

The renal tubular and glomerular structures in CTR, VEH and AP groups had normal histological appearance. In the MTX group, dilatation of Bowman's space accompanying atrophy was observed in most glomeruli. Dilatation in some tubules, hydropic degeneration and atrophy were observed in tubule cells. In addition, the lumen of the proximal convoluted tubules appeared relatively more empty, suggesting a possible reduction in the fringed appearance of the brush border (Table 2 and Figure 4). Compared with the MTX group, the APM group exhibited significantly lower histopathological scores for glomerular atrophy ($p < 0.001$), Bowman's space dilatation ($p < 0.01$), hydropic degeneration of tubular epithelial cells ($p < 0.01$), and tubular atrophy

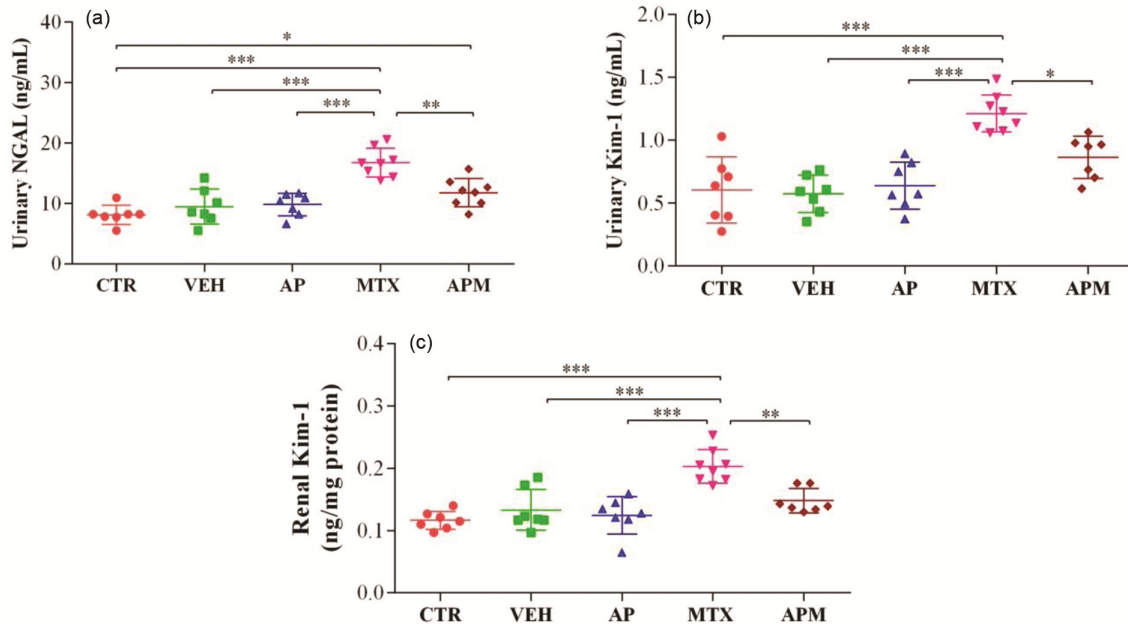


Figure 2 — Urinary NGAL (A), urinary Kim-1 (B), and renal tissue Kim-1 (C) levels in all groups. Intergroup statistical differences are indicated as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

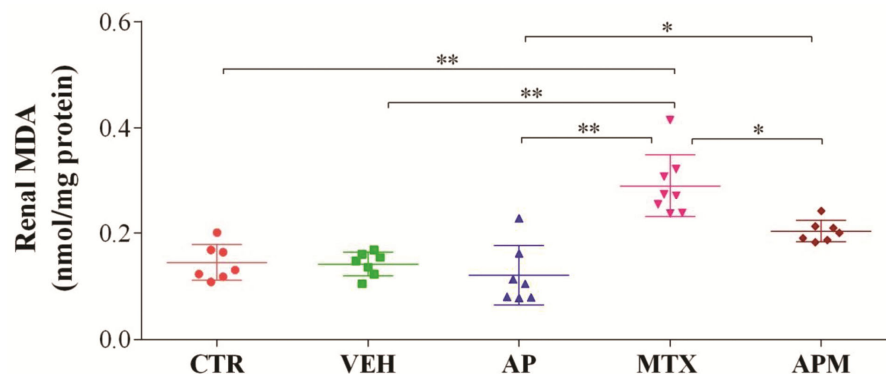


Figure 3 — MDA levels in kidney tissue. Intergroup statistical differences are indicated as * $p < 0.01$ and ** $p < 0.001$.

Table 2 — Scoring of histopathological changes induced by methotrexate, alpha-pinene and vehicle treatments in kidney tissue

Groups	Glomerular atrophy	Dilatation of Bowman's space	Dilatation of tubules	Hydropic degeneration of tubule cells	Tubular atrophy
CTR	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
VEH	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
AP	0 ± 0	0.143 ± 0.38	0 ± 0	0 ± 0	0.143 ± 0.38
MTX	2.875 ± 0.35 * # ⊥	2.750 ± 0.46 * # ⊥	1.750 ± 0.46 * # ⊥	1.750 ± 0.46 * # ⊥	1.875 ± 0.35 * # ⊥
APM	0.125 ± 0.35 φφ	0.250 ± 0.46 φ	0.625 ± 0.52	0.250 ± 0.46 φ	0.125 ± 0.35 φφ

Histopathological scoring: 0, no structural change; 1, slight structural change; 2, moderate structural change; 3, significant structural change. Significant differences compared with the CTR group: * $p < 0.001$; compared with the VEH group: * $p < 0.001$; compared with the AP group: ⊥ $p < 0.001$; and compared with the MTX group: φ $p < 0.01$, φφ $p < 0.001$.

($p < 0.001$). In fact, apart from mild tubular dilatation observed in some tubules, no other histological abnormalities were detected in the APM group (Table 2 and Figure 4).

DISCUSSION

Present study examined the capability of alpha-pinene to act as a protective agent during methotrexate related renal damage. The results demonstrated that two weeks pre-treatment with alpha-pinene successfully reduced the nephrotoxic effects of methotrexate.

Previous experiments conducted in rats have extensively detailed methotrexate-related renal injury using diverse biochemical markers and histopathological analyses⁴⁻⁸. In the present study, histopathological analysis was used for the initial confirmation of methotrexate-induced renal injury, which was subsequently supported by biochemical analysis. Our results revealed glomerular atrophy and dilation of Bowman's space, as well as significant degeneration and loss of tubular cell structure, in the methotrexate injected group. These histological findings indicate that methotrexate causes renal damage at both the glomerular and tubular levels. These results are in line with the literature reporting structural changes induced by methotrexate such as tubular degeneration, necrosis, impaired glomerular integrity, parenchymal collapse and leukocyte infiltration^{6-8, 30}. Although methotrexate induced changes including inflammation, oedema and cellular damage could affect tissue weight, our results show no substantial difference in renal index between the groups. On the other hand, an important decrease in the daily food consumption and body weight gain was detected in animals treated with methotrexate. These results align with previous studies and are likely due

to gastrointestinal toxicity of methotrexate, which can lead to nausea, loss of appetite, mucosal damage and malabsorption^{31, 32}.

In this study, evidence including elevated serum creatinine and BUN concentrations, decreased GFR and increased serum cystatin C levels further supported methotrexate-induced renal injury, with the latter being a more sensitive marker of renal function. Renal damage was also assessed by monitoring urinary biomarkers, with methotrexate administration resulting in a substantial elevation on urinary NGAL and Kim-1 concentrations.

Among these, NGAL is a protein whose expression increases during ischemic and nephrotoxic insults, and elevated urinary concentrations of NGAL serve as an indicator of tubular damage^{33, 34}. Consistent with earlier studies reporting that methotrexate administration (20-80 mg/kg) leads to elevated NGAL levels accompanied by tubular necrosis, cellular degeneration and leukocyte infiltration^{35, 36}, our findings also demonstrated a methotrexate-related rise in urinary NGAL concentrations.

Kim-1, another protein that we examined, is an important biomarker of tubular damage associated with nephrotoxic insults^{34, 37}. Following methotrexate injection in experimental rat models, it has been reported that Kim-1 levels increased in both renal tissue and serum^{38, 39}. A remarkable increase in Kim-1 concentrations in urine has also been observed in children receiving methotrexate treatment, and Kim-1 has been proposed as a valuable marker for the initial identification of acute kidney damage^{34, 40}. In line with the earlier literature, our study found that administering methotrexate significantly increased Kim-1 concentrations in both renal tissue and urine. Increases in both NGAL and Kim-1 concentrations indicate tubular damage, consistent with the histological

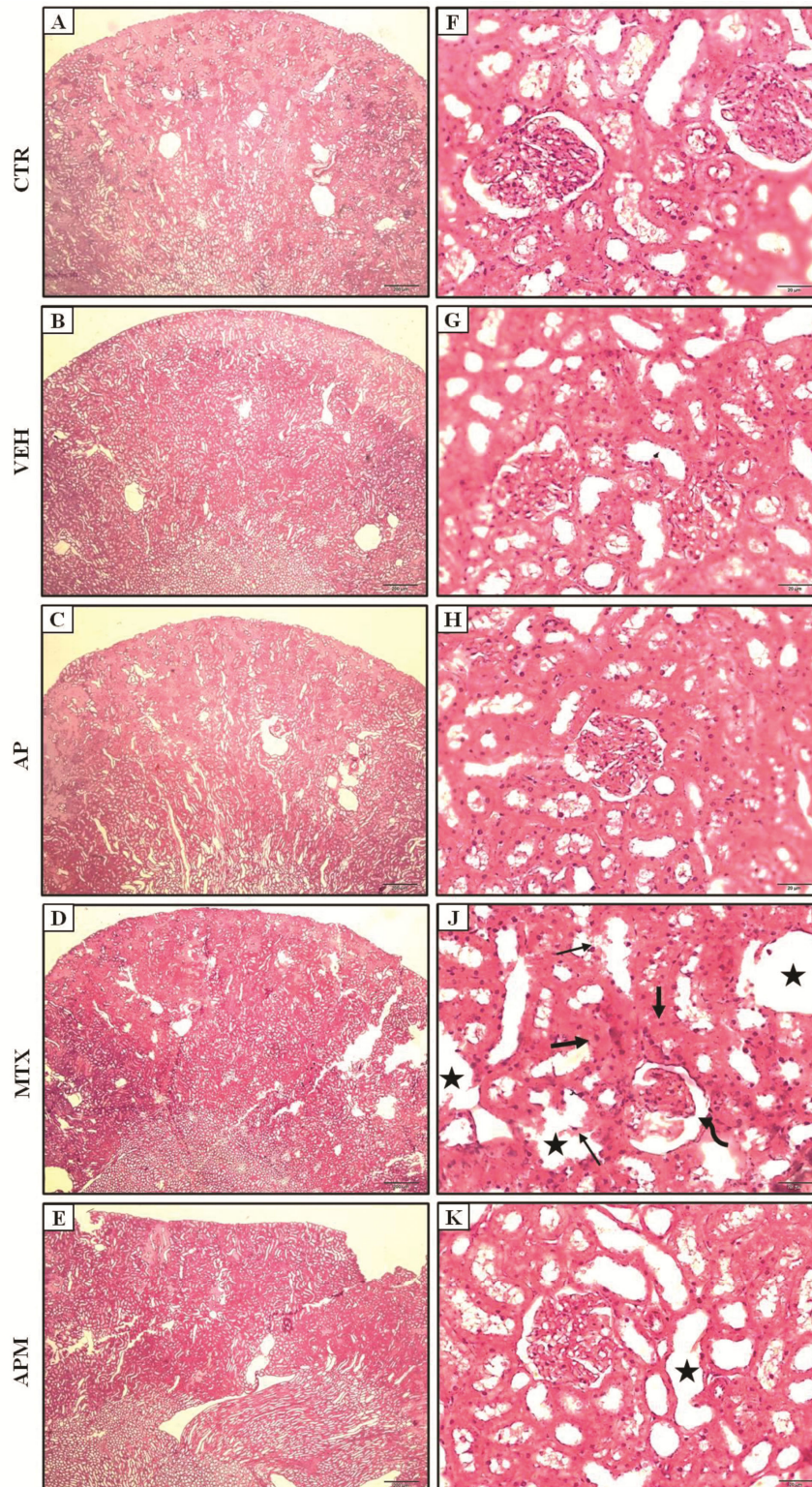


Figure 4 — Representative histological images of kidney tissues from each group. H&E staining, light microscopy, 4x (A–E) and 40x (F–K) magnification. Scale bars: 200 μm (4x magnification) and 20 μm (40x magnification). The broken arrow indicates dilation of Bowman's space; the stars, dilation of the tubules; the thick arrows, hydropic degeneration of the tubular cells; and the thin arrows, tubular atrophy.

findings of tubular injury and an apparent reduction in the fringed appearance of the brush border in the proximal convoluted tubules observed on H&E-stained sections.

This study focuses on the capacity of alpha-pinene to prevent methotrexate-induced kidney damage. Our results demonstrate that alpha-pinene by itself did not induce significant changes in the evaluated parameters in the AP group. In the group that received alpha-pinene pretreatment followed by methotrexate (APM), the general parameters (food and water consumption, weight gain and renal index) were comparable to those of the MTX group. However, improvements in renal function were detected. Compared to the MTX group, BUN, creatinine and cystatin C levels were lower and GFR values were higher in this group. Furthermore, the increase in tubular injury markers (NGAL and Kim-1) observed with MTX group was significantly reduced in the APM group. The absence of significant glomerular and tubular damage apart from minimal tubular dilation in the APM group is consistent with these results. Taken together, our findings indicate, for the first time, that alpha-pinene may offer protection against methotrexate-induced loss of renal function, particularly tubular damage.

Previous studies demonstrating the renoprotective effects of alpha-pinene in experimental models of nephrotoxicity induced by CCl₄ and cisplatin, as well as in ischemia-reperfusion injury, are consistent with present findings^{18, 19, 26, 27}. Unlike these earlier studies, the present work evaluated more sensitive biomarkers, including cystatin C, NGAL, and Kim-1, which have not been thoroughly investigated in the context of alpha-pinene-mediated renoprotection. In this regard, our study makes an important contribution to existing literature by demonstrating the protective effects of alpha-pinene against renal injury, using more specific and sensitive parameters. Notably, we also observed a reduction in tissue lipid peroxidation levels in the APM group, in line with previous reports, supporting the notion that the antioxidant properties of alpha-pinene play a key role in its renoprotective action. Nevertheless, the precise mechanism of alpha-pinene induced protection remains incompletely understood, particularly regarding its anti-inflammatory and anti-apoptotic potential, which is a limitation of the present study. Further studies are needed to explore the involvement of NF- κ B, Nrf2, and apoptosis-related signalling pathways.

Evaluating the biochemical and histopathological data together reveals that alpha-pinene exhibits protective effects during methotrexate-induced renal damage. However, the fact that not all of the parameters examined have returned to the levels observed in the CTR or VEH group suggests that pre-treatment with alpha-pinene may not be sufficient to preserve renal function completely. For instance, higher BUN, serum creatinine and cystatin C levels and lower GFR in the APM group compared to the VEH group, indicate that renal function was not entirely maintained. Similarly, urinary NGAL levels failing to return to control levels despite alpha-pinene pretreatment, suggests ongoing tubular damage. Conversely, the approach of Kim-1 concentrations to the control levels in both urine and tissue samples suggest that the proximal tubules in particular may have benefited significantly from alpha-pinene pretreatment. The difference in results between two tubular injury markers, NGAL and Kim-1, may be related to the fact that NGAL is released not only from tubular cells, but also from active neutrophils that infiltrate the tissue as a result of the inflammatory response induced by methotrexate. The significant reduction in tubular damage following alpha-pinene pretreatment, as demonstrated by histological examination, supports this view.

CONCLUSION

In conclusion, present results demonstrated the renoprotective effectiveness of alpha-pinene from the toxicity caused by methotrexate. By evaluating renal functions and tissue injury using both conventional parameters and specific biomarkers, the study contributed important new insights to the existing literature. These findings, together with the reported anti-cancer properties of alpha-pinene, suggest a potential synergistic therapeutic strategy. Co-administrations of alpha-pinene with methotrexate may offer renal protection while presenting or even enhancing anti-tumour efficacy, thereby supporting the development of dose-optimization or dose-reduction protocols. However, further comprehensive studies are needed to elucidate the molecular mechanisms underlying these renoprotective effects, confirm possible anti-tumour synergy, and determine the optimal dose and treatment duration.

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