Complementary Medicine Research
Practice | Methods | Perspectives

Original Article · Originalarbeit

Complement Med Res
DOI: 10.1159/000468984

Published online: May 4, 2017

© Free Author Copy - for personal use only
ANY DISTRIBUTION OF THIS ARTICLE WITHOUT WRITTEN CONSENT FROM S. KARGER AG, BASEL IS A VIOLATION OF THE COPYRIGHT.
Written permission to distribute the PDF will be granted against payment of a permission fee, which is based on the number of accesses required.
Please contact permission@karger.com

Methotrexate-Induced Nephrotoxicity in Rats: Protective Effect of Mistletoe (*Viscum album* L.) Extract

Esin Sakalli Çetin[a] Hasan Tetiker[b] Özgür İlhan Çelik[c] Nigar Yılmaz[d] İbrahim Hakkı Ciğerci[e]

[a] Department of Medical Biology, Faculty of Medicine, Muğla Sıtkı Kocman University, Muğla, Turkey;
[b] Department of Anatomy, Faculty of Medicine, Muğla Sıtkı Kocman University, Muğla, Turkey;
[c] Department of Pathology, Faculty of Medicine, Muğla Sıtkı Kocman University, Muğla, Turkey;
[d] Department of Biochemistry, Faculty of Medicine, Muğla Sıtkı Kocman University, Muğla, Turkey;
[e] Department of Molecular Biology and Genetics, Faculty of Science, Afyon Kocatepe University, Afyon, Turkey

Keywords
Mistletoe · Methotrexate · Oxidative stress · Nephrotoxicity · Comet assay

Summary
Background: The protective effect of mistletoe extract (Helixor®, HLX) against methotrexate (MTX)-induced acute oxidative stress and nephrotoxicity in rats was evaluated by histological and biochemical methods as well as the comet assay. **Material and Methods:** 32 female Wistar albino rats were divided into 4 groups: control group, HLX group (5 mg/kg body weight (bw), days 1–10, intraperitoneally (i.p.)), MTX group (10 mg/kg bw, days 7, 8, and 9, i.p.), and MTX + HLX group (10 mg/kg bw, days 7, 8, and 9, i.p. + 5 mg/kg bw, days 1–10, i.p.). At the end of the experiment, the glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), nitric oxide (NO), and myeloperoxidase (MPO) levels were measured, and a histopathological analysis and comet assay were carried out. **Results:** MTX induced renal oxidative stress and nephrotoxicity in the rats. Pretreatment with HLX significantly improved the renal GSH-Px and SOD activities in the MTX + HLX group compared to the MTX group. The decrease in the NO and MPO levels in the rat groups pretreated with HLX was not significant. The histochemical evaluation revealed that HLX provided significant improvement in the MTX-induced renal degenerative changes, including tubule distension, interstitial inflammation, perirenal inflammation, glomerular congestion, glomerular degeneration, and parenchymal hemorrhage, in the MTX + HLX group compared to the MTX-administered group. According to the comet assay, pretreatment with HLX lowered the MTX-induced DNA damage in endogenous lymphocytes, although not significantly. **Conclusion:** This study demonstrated that HLX administration markedly reduced the MTX-induced acute oxidative stress and nephrotoxicity in rats through its antioxidant and anti-inflammatory properties.

© 2017 S. Karger GmbH, Freiburg

Schlüsselwörter
Mistel · Methotrexat · Oxidativer Stress · Nephrotoxizität · Comet-Assay

Zusammenfassung
Hintergrund: Der Schutzeffekt von Mistelextrakt (Helixor®, HLX) gegen Methotrexat (MTX)-induzierten akuten oxidativen Stress und Nephrotoxizität bei Ratten wurde mit histologischen und biochemischen Methoden sowie dem Comet-Assay evaluiert. **Material und Methoden:** 32 weibliche Wistar-Albino-Ratten wurden in 4 Gruppen eingeteilt: Kontrollgruppe, HLX-Gruppe (5 mg/kg Körpergewicht (bw), Tag 1–10, intraperitoneal (i.p.)), MTX-Gruppe (10 mg/kg bw, Tag 7, 8, und 9, i.p.), und MTX + HLX-Gruppe (10 mg/kg bw, Tag 7, 8, und 9, i.p. + 5 mg/kg bw, Tag 1–10, i.p.). Am Ende des Experiments wurden die Glutathion-Peroxidase (GSH-Px)-, Superoxid-Dismutase (SOD)-, Stickoxid (NO)- und Myeloperoxidase (MPO)-Spiegel gemessen; eine histopathologische Analyse und ein Comet-Assay wurden durchgeführt. **Ergebnisse:** MTX führte bei den Ratten zu oxidativem Stress in der Niere und zu Nephrotoxizität. Eine Vorbehandlung mit HLX führte bei der MTX + HLX-Gruppe im Vergleich zur MTX-Gruppe zu einer signifikanten Verbesserung der renalen GSH-Px- und SOD-Aktivitäten. Der Abfall der NO- und MPO-Spiegel in den mit HLX vorbehandelten Rattengruppen war nicht signifikant. Die histochemische Untersuchung ergab, dass HLX in der MTX + HLX-Gruppe im Vergleich zur MTX-Verabreichungsgruppe eine signifikante Verbesserung der MTX-induzierten degenerativen Nierenveränderungen hervorrief, einschließlich der Tubulierweiterung, der interstitiellen Entzündung, der perirenalen Entzündung, der glomerulären Blutstauung, der glomerulären Degeneration und der parenchymalen Blutung. Basierend auf dem Comet-Assay erniedrigt eine Vorbehandlung mit HLX die MTX-induzierten DNA-Schäden in den endogenen Lymphozyten, allerdings nicht signifikant. **Schlussfolgerung:** Diese Studie zeigt, dass die Gabe von HLX aufgrund seiner antioxidativen und antientzündlichen Eigenschaften den MTX-induzierten akuten oxidativen Stress und die Nephrotoxizität bei Ratten deutlich reduziert.

KARGER

Fax +49 761 4 52 07 14
Information@Karger.com
www.karger.com

© 2017 S. Karger GmbH, Freiburg

Accessible online at:
www.karger.com/cmr

Prof. Dr. Esin Sakalli Çetin
Department of Medical Biology, Faculty of Medicine
Muğla Sıtkı Kocman University
Muğla, Turkey
esincetin@mu.edu.tr

Introduction

Methotrexate (MTX) is a well-known chemotherapeutic agent and it is used in the treatment of autoimmune diseases due to its anti-inflammatory and immunosuppressive effects [1]. Despite the spectrum of clinical use, the efficacy of MTX is often limited by severe adverse effects, mainly nephrotoxicity and hepatotoxicity; it also has other side effects such as intestinal injury and myelosuppression [2]. As MTX is primarily cleared by the kidneys, both the precipitation of MTX in the kidney tubules and glomerular filtration rate decreases cause kidney injury at high doses of MTX. The risk of kidney toxicity is 2% in patients with MTX treatment [3]. Although the exact pathogenesis of MTX-induced nephrotoxicity is not understood, enhancement of the formation of reactive oxygen species (ROS), neutrophil infiltration, inhibition of DNA synthesis, and release of inflammatory mediators including interleukin (IL)-1β and tumor necrosis factor (TNF)-α are reported to play important roles [2, 4–6]. Increased expression of TNF-α, a key modulator in liver and kidney homeostasis, was reported in a model of MTX-induced hepatic, renal, and intestinal damage [6, 7]. The pro-inflammatory effects of TNF-α are mediated through nuclear factor kappa B (NF-κB)-regulated proteins, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [8].

In the present study, a mistletoe preparation was selected due to its anti-inflammatory effects, which inhibit cytokine-induced secretion of prostaglandin E2, an important molecular mediator of inflammatory reactions, by selectively inhibiting COX-2 [9, 10]. Its selection was also based on its antioxidant properties, which have been previously reported to prevent oxidative damage [11–17].

Mistletoe (*Viscum album* L.), a semiparasitic plant, grows on different host trees. *V. album* preparations, including Helixor®, Iscador®, Isorel®, Plenosol®, and Iscucin®, are standardized aqueous extracts of mistletoe [18–22]. They are composed mainly of mistletoe lectins and viscotoxins and other molecules, such as polysaccharides, flavonoids, thiols, cyclitols, phytosterols, and triterpenes, depending on the harvesting time and host tree [18–22]. *V. album* preparations have been used as complementary therapies in cancer, in addition to conventional treatments. When utilized with standard chemotherapy or radiotherapy, *V. album* preparations contribute to a significant improvement in the patient's quality of life [23]. Reported effects of *V. album* preparations on tumors include not only the induction of tumor cell apoptosis and inhibition of angiogenesis but also the modulation of the immune system, exerting a potent anti-inflammatory effect, and protection of the DNA of healthy cells against damage caused by cytostatic drugs [24–26]. As a direct result, the side effects of chemotherapy and radiotherapy are reduced [24–26].

Several studies demonstrated that various agents, including caffeic acid phenethyl ester [5], melatonin [27], curcumin [28], thymoquinone [29], pentoxifylline [2, 30], and alpha-lipoic acid [30], had beneficial effects helping to reduce MTX-induced tissue damage. Considering the nephrotoxic and genotoxic effects of MTX, we hypothesized that a *V. album* preparation (Helixor® (HLX)) may improve MTX-induced oxidative stress and nephrotoxicity. To date, the effects of HLX on MTX-induced nephrotoxicity have not been studied. Thus, the aim of this study was to investigate the effect of HLX on MTX-induced oxidative stress and nephrotoxicity by using biochemical methods, histological examinations, and molecular methods.

Materials and Methods

Chemicals

MTX was purchased from Koçak Pharma Drug and Chemical Industry Co. Ltd. Helixor M (lot 4112505) was purchased from Helixor Heilmittel GmbH & Co KG, Rosenfeld, Germany. The vial of Helixor M contained 50 mg total plant extract of *V. album* L. in 1 ml water.

Animals

The experimental procedures and the protocols for animal use were approved by the Animal Ethics Committee of the Süleyman Demirel University, Isparta, Turkey (No. 28.08.2012/05). 32 female Wistar albino rats, each weighing 200–220 g, were purc hased and maintained in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals set up by the Süleyman Demirel University, Isparta, Turkey.

Experimental Protocol

The dose of HLX was selected as 5 mg/kg because previous studies [31, 32] had demonstrated that complementary treatment with HLX can beneficially reduce the side effects of chemotherapy and improve the quality of life in cancer patients at doses of 1–500 mg/kg body weight (bw). We performed a preliminary experiment with different doses determined on the basis of dose translation from human dosage to animal dosage [33].

The experimental rats were further randomly divided into 4 subgroups with 8 rats in each group;

- Group I. Control group: The rats were intraperitoneally (i.p.) injected with isotonic saline solution.
- Group II. HLX group: The rats were i.p. injected with Helixor M (5 mg/kg bw, on days 1–10).
- Group III. MTX group: The rats were i.p. injected with MTX (10 mg/kg bw, on days 7, 8, and 9) [34].
- Group IV. MTX + HLX group: The rats were i.p. injected with Helixor M (5 mg/kg bw, on days 1–10) and MTX (10 mg/kg bw, on days 7, 8, and 9).

Specimen Collection

At the end of the experiment, the rats were anesthetized with intramuscular ketamine hydrochloride (Ketalar, 50 mg/kg; Eczacibasi, Istanbul, Turkey), venous blood samples were taken, and the sera were separated after centrifugation at 4,000 rpm for 5 min at 4°C. Then, both kidneys were rapidly excised and the left kidneys were equally divided into 2 longitudinal pieces. One half of the left kidney was placed in formaldehyde solution for routine histopathological examination, and the entire right kidney and the other half of the left kidney were washed with physiological saline for biochemical analyses. The kidney tissue samples were stored at –80°C until analysis.

Biochemical Analysis

Measurement of Renal SOD Activity

Tissue samples were homogenized with ice-cold buffer containing 20 mM HEPES buffer (pH 7.4), 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose per gram tissue for 2 min at 16,000 rpm and 4°C. Then, the homogenate was centrifuged at 1500 × g for 5 min at 4°C to remove the debris. The clear supernatant fluid was collected to carry out the SOD activity assays. Analysis of the SOD activity was performed with Cayman's Superoxide Dismutase Assay Kit (Cayman Chemical Co., Ann Arbor, MI, USA) and read out in a Bio-Tek ELx-800 (Winooski, VT, USA) absorbance reader. The SOD activity was expressed

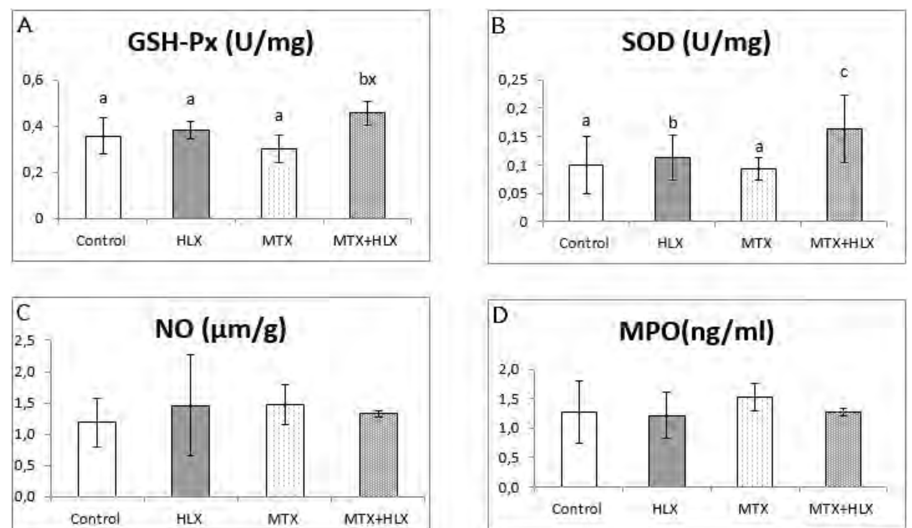

Fig. 1. Renal (**A**) GSH-Px activity, (**B**) SOD activity, (**C**) NO levels, (**D**) MPO levels in the control, HLX, MTX, and MTX + HLX groups. Each group consists of 8 animals. Different characters (a, b, c) above the columns represent significance at $p < 0.05$; 'x' represents significance at $p < 0.001$. GSH-Px = glutathione peroxidase, SOD = superoxide dismutase, NO = nitric oxide, MPO = myeloperoxidase, HLX = Helixor, MTX = methotrexate.

as units per milligram protein for tissue and units per milliliter for serum. 1 U of SOD was described as the amount of enzyme causing 50% inhibition in the nitro blue tetrazolium (NBT) reduction rate by the xanthine-xanthine oxidase system as a superoxide generator.

Measurement of Renal GSH-Px Activity

Tissue samples were homogenized with ice-cold buffer (50 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 1 mM dithiothreitol (DTT)) per gram tissue at 4°C. Then, the homogenate was centrifuged at 10,000 × g for 15 min at 4°C to remove the debris. The clear supernatant fluid was collected to determine the GSH-Px activities. The GSH-Px activity analysis was performed with Cayman's GSH-Px Assay Kit (Cayman Chemical Co.) and read out in a Bio-Tek ELx-800 absorbance reader. The principle of the method relies on the detection of nicotinamide adenine dinucleotide phosphate (NADPH) oxidation by hydrogen peroxide, at 340 nm. 1 U of GSH-Px activity is defined as the amount of enzyme needed to oxidize 1 nmol of NADPH per minute.

Measurement of the Renal NO Levels

The tissue was homogenized in phosphate-buffered saline (PBS; pH = 7.4) and centrifuged at 10,000 × g for 15 min at 4°C. The principle of the method is based, briefly, on measuring the total nitrite by spectrophotometry at 545 nm in a Bio-Tek ELx-800, after conversion of nitrate into nitrite, by using a nitrate/nitrite colorimetric assay kit (Cayman Chemical Co.). A standard curve was established from nitrite standards to analyze unknown sample concentrations, and the NO level was expressed as μM/g protein.

Measurement of the Renal MPO Levels

Tissue-associated MPO activity was determined by an enzyme-linked immunosorbent assay kit (MPO Instant ELISA; eBioscience, Vienna, Austria) and measured in a Bio-Tek ELx-800. 1 U of enzyme activity is expressed as ng/ml protein.

Histological Evaluation

For the light-microscopic evaluation, renal tissues were fixed in 10% formaldehyde and processed routinely for embedding in paraffin. Tissues were sectioned into 4-μm-thick slices, stained with hematoxylin and eosin (H&E) and examined under an Olympus BX51 (Tokyo, Japan) photomicroscope.

Renal injury was evaluated based on 10 sections per rat kidney at 100–400 × magnification (assessed by an examiner who did not know the treatment group) according to the following criteria: (1) distension of the tubules, (2) interstitial inflammation, (3) perirenal inflammation, (4) glomerular congestion, (5) glomerular degeneration, (6) parenchymal hemorrhage, and (7) perirenal eosinophil infiltration. Each criterion was scored (1 score per rat) using a semiquantitative scale as follows: 0 = none, 1 = mild, 2 = moderate, 3 = severe [35–37].

Comet Assay

The comet assay (alkaline single-cell gel electrophoresis assay) was used to determine the endogenous lymphocyte DNA damage occurring as single-strand breaks, by measuring the migration of DNA fragments from the nucleoid, visually resembling a comet. 100 randomly chosen nuclei per rat (50 cells analyzed in each slide) were examined at 400 × magnification using a fluorescence microscope (Olympus, Japan). Each image was classified according to the intensity of the fluorescence in the comet tail and was given a value of 0, 1, 2, 3, or 4, so that the total scores of the slide amounted to between 0 and 400 arbitrary units (AU).

Statistical Analysis

Statistical evaluations were performed using the program SPSS 20.0 for Windows. In general, any significant differences between these groups were evaluated using the Kruskal-Wallis test. The Mann-Whitney U test was used to compare the groups with each other. Results are presented as mean + standard deviation (SD); $p < 0.05$ was regarded as statistically significant; $p < 0.01$ was regarded as highly statistically significant.

Results

NO, MPO Levels and GSH-Px, SOD Activities in the Kidney

Both table 1 and figure 1 summarize the results. All rats survived without major complications.

The renal GSH-Px activity was found to be significantly higher in the MTX + HLX group compared to both the control group ($p < 0.05$) and the MTX group ($p < 0.001$). However, it was found to be increased in the HLX group and decreased in the MTX group compared to the control group, albeit not significantly ($p > 0.05$) (fig. 1A).

The renal SOD activity was significantly increased in the HLX + MTX group compared with the control group ($p < 0.05$) and the MTX group ($p < 0.05$). The SOD values of the HLX group compared to the control group were increased, and the values in the MTX group were decreased, but these values were not significantly different when compared to the control group ($p > 0.05$) (fig. 1B).

The renal NO values in the MTX group compared to the control group were found to be increased, and pretreatment with HLX decreased the values in the MTX + HLX group, but not significantly ($p > 0.05$). However, no significant difference was found between the MTX group and the MTX + HLX group ($p > 0.05$) (fig. 1C).

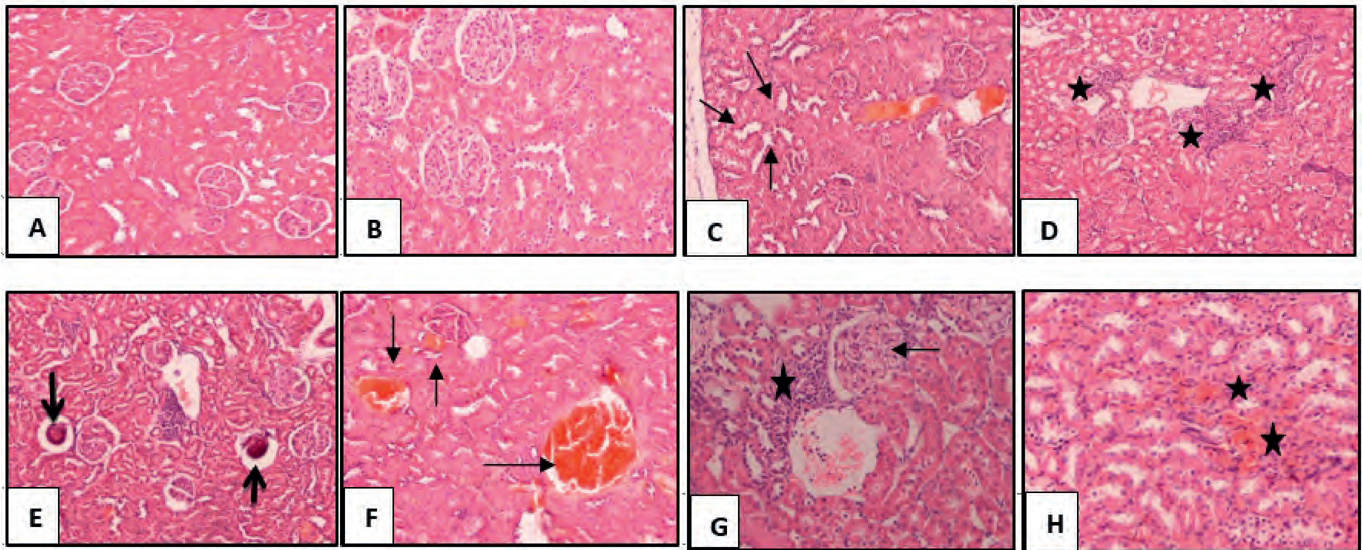

Fig. 2. (**A**) Control group (H&E, × 40): normal renal morphology, (**B**) HLX group (H&E, × 100): normal renal morphology, (**C**) MTX group (H&E, × 40): distension of tubules (arrows), (**D**) MTX group (H&E, × 40): interstitial inflammation (stars), (**E**) MTX group (H&E, × 200): glomerular degeneration (arrows), (**F**) MTX group (H&E, × 200): parenchymal hemorrhage (arrows), (**G**) MTX + HLX group (H&E, × 200): mild interstitial inflammation (stars) and minimal glomerular congestion (arrows), (**H**) MTX + HLX group (H&E, × 200): mild parenchymal hemorrhage (stars). H&E = hematoxylin and eosin, HLX = Helixor, MTX = methotrexate.

Table 1. GSH-Px, SOD, NO, and MPO values in the kidneys of the 4 groups of rats (n = 8 each)

	Control	HLX	MTX	MTX + HLX
GSH-Px, U/mg	0.35713 ± 0.08^{a}	0.38250 ± 0.04^{a}	0.30037 ± 0.06^{a}	0.45538 ± 0.05^{bx}
SOD, U/mg	0.10023 ± 0.05^{a}	0.11246 ± 0.04^{b}	0.09363 ± 0.02^{a}	0.16344 ± 0.06^{c}
NO, μm/g	1.18576 ± 0.39	1.45688 ± 0.81	1.47944 ± 0.32	1.32408 ± 0.05
MPO, ng/ml	1.26298 ± 0.53	1.21612 ± 0.39	1.52706 ± 0.24	1.27288 ± 0.07

Results are presented as mean ± SD. Groups of data were compared with the Kruskal-Wallis test followed by the Mann-Whitney U test.
GSH-Px = Glutathione peroxidase, SOD = superoxide dismutase, NO = nitric oxide, MPO = myeloperoxidase, SD = standard deviation.
Values followed by different characters (a, b, c) in the columns are significantly different at $p < 0.05$.
Values followed by the 'x' character in the columns are significantly different at $p < 0.001$.

Table 2. Histopathological findings in the kidneys of the 4 groups of rats (n = 8 each)

Histopathologic parameters	Control	HLX	MTX	MTX + HLX
Tubular distension	0.000^{a}	0.50^{b}	1.5^{cx}	0.75^{d}
Interstitial inflammation	0.000^{a}	0.125^{b}	1.5^{cx}	0.125^{bx}
Perirenal inflammation	0.000^{a}	0.125^{b}	0.625^{cx}	0.000^{a}
Glomerular congestion	0.000^{a}	0.000^{a}	1.125^{bx}	0.625^{c}
Glomerular degeneration	0.000^{a}	0.000^{a}	1.125^{bx}	0.25^{c}
Parenchymal hemorraghe	0.000^{a}	0.000^{a}	1.25^{bx}	0.000^{a}

Results are presented as the median of the scores. Groups of data were compared with the Kruskal-Wallis test followed by the Mann-Whitney U test.
HLX = Helixor, MTX = methotrexate.
Values followed by different characters (a, b, c, d) in the columns are significantly different at $p < 0.05$.
Values followed by the 'x' character in the columns are significantly different at $p < 0.001$.

The renal MPO values were found to be increased in the MTX group and decreased in the HLX group compared to the control group, but not significantly ($p > 0.05$). Pretreatment with HLX decreased the MPO values in the MTX + HLX group; however, no significant difference was found between the MTX group and the MTX + HLX group ($p > 0.05$) (fig. 1D).

Kidney Histopathology

Table 2 summarizes the kidney histopathology results of all groups. In the histologic examination, the kidney tissues of the control and HLX groups showed normal kidney morphology (fig. 2A and 2B). MTX significantly caused tubular distension ($p = 0.000$) (fig. 2C), interstitial inflammation ($p = 0.003$) (fig. 2D), per-

Table 3. Comet assay values of the 4 groups of rats

	Control	HLX	MTX	MTX + HLX
DNA damage, AU ± SD*	15.00 ± 3.00[a]	19.00 ± 3.60[ab]	31.00 ± 7.00[c]	26.66 ± 2.30[bc]

*Mean ± SD.
HLX = Helixor, MTX = methotrexate, AU = arbitrary unit, SD = standard deviation.
Values followed by different characters (a, b, c, d) are significantly different at $p < 0.05$ (Duncan test).

irenal inflammation (p = 0.009), glomerular congestion (p = 0.000), glomerular degeneration (p = 0.001) (fig. 2E), and parenchymal hemorrhage (p = 0.004) (fig. 2F).

Moreover, administration of HLX plus MTX provided a great improvement regarding the tubule distension (p = 0.015) (fig. 2G), the interstitial inflammation (p = 0.009), the perirenal inflammation (p = 0.009), the glomerular congestion (p = 0.044) (fig. 2G), the glomerular degeneration (p = 0.011), and the parenchymal hemorrhage (p = 0.004) (fig. 2H); these values were found to be statistically significant compared to the MTX group.

Comet Assay Results

The comet assay results showed that the DNA damage was higher in the MTX group compared to the control group. While the highest genotoxic activity was observed in the MTX group (31.00 ± 7.00), the lowest one was observed in the control group (15.00 ± 3.00). The decrease in DNA damage in the MTX + HLX group is statistically non-significant compared to that of the MTX group. The DNA damage in the MTX group is significantly higher than that in the control and HLX group, but no significant differences could be seen between MTX + HLX or MTX alone (table 3).

Discussion

In the present study, mistletoe extract clearly exerted a protective effect against MTX-induced oxidative stress, inflammatory cell infiltration, and nephrotoxicity in rats due to its powerful antioxidant and anti-inflammatory properties. The results showed that MTX caused oxidative renal tissue damage, as evidenced by renal histopathological findings in the form of tubular distension, interstitial inflammation, perirenal inflammation, glomerular congestion, glomerular degeneration, parenchymal hemorrhage, and perirenal eosinophil infiltration, which is in agreement with the findings of previous studies [35–37]. Pretreatment with HLX before the administration of MTX ameliorated the MTX-induced damage of the kidneys. The exact mechanism of MTX-induced nephrotoxicity remains obscure. However, some studies demonstrated that the main factor in MTX-associated tissue injury was oxidative damage, with subsequent free-radical generation [4–6]. The role of oxidative stress has been documented in MTX-induced nephrotoxicity [2, 5, 6, 27–30, 38] and hepatotoxicity [6, 29, 30]. Here, we showed for the first time that HLX ameliorated MTX-induced oxidative stress and nephrotoxicity. The mechanism included reversing the MTX-induced renal oxidative stress, as indicated by the significant increase in GSH-Px and SOD activities. The decrease in the NO and MPO levels in the rat groups pretreated with HLX was not significant. HLX lowered, although not significantly, the MTX-induced DNA damage in endogenous lymphocytes.

NO is a free radical formed from L-arginine by NOS. The overproduction of NO, which reacts with superoxide anions, leads to the formation of peroxynitrite ($ONOO^-$). Peroxynitrite oxidizes cellular structures and causes lipid peroxidation and ROS formation, resulting in cellular injury. It has been reported that increased peroxynitrite caused renal injury and damage to arteries and tubules [39]. Previous studies showed that an MTX overdose led to nephrotoxicity due to lipid peroxidation, which resulted in increased levels of malondialdehyde (MDA), NO release, and ROS formation [2, 5, 6, 27–30]. Similarly, in the present study, the level of NO was increased in the kidney tissues of the MTX group. It was also elevated in the HLX group. *V. album* extract exerts a positive effect on cardiac tissue via its vasodilatory activity, which is mediated by increases in NO production. NO formation in vascular endothelial cells modulates the vasodilator tone, and it is necessary for the regulation of blood flow and pressure. Tenorio et al. [40] and Tenorio-Lopez et al. [41] reported that a *V. album*-induced increase in cardiac NO levels had hypotensive and vasodilatory effects in an isolated and perfused heart model. Similarly, elevated NO levels following *V. album* administration in rats were demonstrated in heart tissue [42]. However, in the present study, HLX administration decreased the MTX-induced NO levels, although not significantly. Korean mistletoe (*V. album coloratum*) lectin was reported to exert an immunomodulatory effect by blocking lipopolysaccharide-induced NO production in macrophage-like cells [43]. The protective effects of Korean mistletoe lectin against oxidative stress were reported to be linked to the down-regulation of mRNA and protein expression of iNOS and COX-2 through NF-κB regulation and inhibition of NO production [17]. Overexpression of pro-inflammatory mediators including TNF-α, NF-κB, COX-2, and iNOS was shown to play an important role in the direct nephrotoxicity effects of MTX [6, 28, 29, 35, 44]. Therefore, we suggest that HLX treatment might prevent the MTX-induced increases in iNOS levels. This suggestion is in agreement with the histological findings in the present study, which demonstrated that the administration of HLX greatly ameliorated the MTX-induced inflammation in renal tissues.

MPO is a heme peroxidase enzyme found in neutrophil primary granules and monocyte lysosomes that leads to tissue damage in acute and chronic inflammation [45]. Thus, inhibiting the enzymatic activity of MPO may be beneficial in the treatment of inflammation-related diseases [45]. In the present study, MTX elevated the MPO activity, pointing to an accumulation of inflammatory

cells (neutrophils and monocytes) in the kidney tissue. This observation is in agreement with the histological findings, which revealed interstitial and perirenal inflammation in the renal tissue of the MTX group. The MPO activity elevation following MTX administration in rats has already been demonstrated earlier, in the kidneys [27, 46] and the liver [46]. The HLX-induced decrease in the MPO activity in this study, although not significant, suggests that inflammatory cell infiltration might be restricted. This protective mechanism appears to be related to the increased NO levels, which inhibit platelet and neutrophil aggregation and therefore mitigate the effects of elevated MPO activity [47]. A previous study demonstrated that *V. album* extract attenuated cyclophosphamide (CP)-induced increases in MPO activity in both heart and bladder tissues [38].

The production of free radicals is prevented by the endogenous antioxidative defense system. SOD and GSH-Px are the main antioxidative enzymes in the cytosol of living cells that protect against ROS-induced oxidative damage. The release of free radicals results in extensive cellular damage when the levels surpass the antioxidative capacity of the biological system. An increase in the activity of antioxidative enzymes has been shown to prevent oxidative stress-associated tissue injury [48]. In the present study, the activities of SOD and GSH-Px decreased in the kidney tissue of the MTX-only group, which is consistent with the findings of recent studies [2, 6, 28, 30]. HLX, a powerful antioxidant, confers protection against MTX-induced toxicity by inhibiting the initiation of oxidative stress. Following the mistletoe administration, the activities of GSH-Px and SOD significantly increased in the HLX + MTX group. The increases in antioxidant enzyme activities may reflect an improved antioxidant status of the rats pretreated with HLX, as indicated by the elevation of the GSH-Px and SOD levels. This observation is in agreement with the findings of an earlier study, which reported that pretreatment with a methanolic extract of European mistletoe (*V. album* L.) increased the antioxidant enzyme activities of catalase, SOD, GSH-Px, and glutathione S-transferases in the heart of a CP + *V. album* group as compared to a CP-only-treated group [34]. Moreover, animal studies reported protective effects of *V. album* extract against oxidative stress in the liver, kidney, brain, and heart of rats [15, 34, 49]. This antioxidant activity of *V. album* extract is associated with its pharmacologically active constituents, mostly flavonoids and lectins, which act as free-radical scavengers, reducing agents, singlet oxygen quenchers, hydrogen donors, and metal chelators [50–52]. Similar results were observed in different studies in which other parasitic plant extracts (mistletoe-like plants) were used. Treatment with a mistletoe alkali, which is a lipid-soluble antioxidant isolated from Chinese mistletoe extract (*V. coloratum* (Komar) Nakai), elevated the GSH-Px and SOD activities in the liver and kidney tissue and in the plasma of rats treated with carbon tetrachloride (CCI_4) [52]. Similarly, the high phenol content of Eastern Nigerian mistletoe (*Loranthus micranthus* Linn.) was responsible for its high antioxidant potential observed in diabetic rats [11]. Furthermore, the antioxidant and hepatoprotective activity of African mistletoe *Tapinanthus bangwensis* (Engl. & K. Krause) in rats was reported to be due to the presence of flavonoids [53]. In a study of Korean mistletoe (*V. album coloratum*) lectin, the authors suggested that it showed radical-scavenging activity and protective effects against oxidative stress induced by free radicals, NO, superoxide anions (O_2^-), and peroxynitrite in vitro [17].

MTX, a folate antagonist, competitively binds to the folate-dependent enzyme dihydrofolate reductase, inhibiting thymidylate synthesis and, hence, DNA synthesis. MTX also causes folate deficiency, which leads to genotoxic damage [1]. In the present study, DNA damage caused by MTX was demonstrated by a comet assay. Previous studies used comet assays to evaluate MTX-induced germ cell toxicity and MTX-induced DNA damage of intestinal cells [1, 54].

In addition to the antitumor activities and chemopreventive effects of *V. album*, antigenotoxic effects of *V. album* have been demonstrated [13, 34, 49]. In the present study, pretreatment of the rats with HLX lowered, although not significantly, the MTX-induced DNA damage in endogenous lymphocytes, as determined by decreased DNA damage values in the comet assay. Similar results were obtained in another study, which reported that *V. album* extract attenuated the cytogenotoxic effects of MTX by reducing the number of chromosomal aberrations and significantly increasing the mitotic index in mouse bone marrow cells [49]. Another study demonstrated similar results in CP-induced mouse bone marrow cells [34]. *V. album* was also reported to protect against H_2O_2-induced oxidative nuclear and mitochondrial DNA damage in vitro, due to its high quercetin content [13].

In conclusion, the present study demonstrated that pretreatment with HLX alleviated the MTX-induced nephrotoxicity in rats via its antioxidant and anti-inflammatory properties, as evident from histopathological improvements and significant increases in the activities of the antioxidative enzymes SOD and GSH-Px. The decrease in the NO and MPO levels in the rat groups pretreated with HLX was not significant. In addition, pretreatment with HLX lowered, albeit not significantly, the MTX-induced DNA damage in endogenous lymphocytes. The improvement in animals pretreated with *V. album* may suggest that further investigations should be performed to explore the beneficial effects of *V. album* to overcome one of the most serious problems in chemotherapy.

Disclosure Statement

The authors declare that there are no conflicts of interest.

Acknowledgement

The present study was supported by the Research Fund of Mugla Sıtkı Koçman University (Project Number 13/11).

© Free Author Copy - for personal use only
ANY DISTRIBUTION OF THIS ARTICLE WITHOUT WRITTEN CONSENT FROM S. KARGER AG, BASEL IS A VIOLATION OF THE COPYRIGHT.
Written permission to distribute the PDF will be granted against payment of a permission fee, which is based on the number of accesses required.
Please contact permission@karger.com

References

1 Padmanabhan S, Tripathi DN, Vikram A, Ramarao P, Jena GB: Methotrexate-induced cytotoxicity and genotoxicity in germ cells of mice: intervention of folic and folinic acid. Mutat Res 2009;673:43–52.

2 Asvadi I, Hajipour B, Asvadi A, Asl NA, Roshangar L, Khodadadi A: Protective effect of pentoxyfilline in renal toxicity after methotrexate administration. Eur Rev Med Pharmacol Sci 2011;15:1003–1009.

3 Widemann BC, Adamson PC: Understanding and managing methotrexate nephrotoxicity. Oncologist 2006;11:694–703.

4 Leitão RF, Brito GA, Oriá RB, Braga-Neto MB, Bellaguarda EA, Silva JV, et al: Role of inducible nitric oxide synthase pathway on methotrexate-induced intestinal mucositis in rodents. BMC Gastroenterol 2011; 11:90.

5 Uz E, Oktem F, Yilmaz HR, Uzar E, Ozgüner F: The activities of purine-catabolizing enzymes and the level of nitric oxide in rat kidneys subjected to methotrexate: protective effect of caffeic acid phenethyl ester. Mol Cell Biochem 2005;277:165–170.

6 Hafez HM, Ibrahim MA, Ibrahim SA, Amin EF, Goma W, Abdelrahman AM: Potential protective effect of etanercept and aminoguanidine in methotrexate-induced hepatotoxicity and nephrotoxicity in rats. Eur J Pharmacol 2015;768:1–12.

7 Cetiner M, Sener G, Sehirli AO, Ekşioğlu-Demiralp E, Ercan F, Sirvanci S, et al: Taurine protects against methotrexate-induced toxicity and inhibits leukocyte death. Toxicol Appl Pharmacol 2005;209:39–50.

8 Aggarwal BB, Gupta SC, Kim JH: Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey. Blood 2012;119:651–665.

9 Hegde P, Maddur MS, Friboulet A, Bayry J, Kaveri SV: *Viscum album* exerts anti-inflammatory effect by selectively inhibiting cytokine-induced expression of cyclooxygenase-2. PLoS One 2011;6:e26312.

10 Saha C, Hegde P, Friboulet A, Bayry J, Kaveri SV: *Viscum album*-mediated COX-2 inhibition implicates destabilization of COX-2 mRNA. PLoS One 2015;10: e0114965.

11 Onunogbo C, Ohaeri OC, Eleazu CO, Eleazu KC: Chemical composition of mistletoe extract (*Loranthus micranthus*) and its effect on the protein, lipid metabolism and the antioxidant status of alloxan induced diabetic rats. J Med Res 2012;1:57–62.

12 Önay-Uçar E, Karagöz A, Arda N: Antioxidant activity of *Viscum album* ssp. *album*. Fitoterapia 2006;77:556–560.

13 Önay-Uçar E, Erol O, Kandemir B, Mertoğlu E, Karagöz A, Arda N: *Viscum album* L. extract protects HeLa cells against nuclear and mitochondrial DNA damage. Evid Based Complement Alternat Med 2012; 2012:958740.

14 Oluwaseun AA, Ganiyu O: Antioxidant properties of methanolic extracts of mistletoes (*Viscum album*) from cocoa and cashew trees in Nigeria. Afr J Biotechnol 2008;7:3138–3142.

15 Orhan DD, Aslan M, Sendogdu N, Ergun F, Yesilada E: Evaluation of the hypoglycemic effect and antioxidant activity of three *Viscum album* subspecies (European mistletoe) in streptozotocin-diabetic rats. J Ethnopharmacol 2005;98:95–102.

16 Kim MS, Lee J, Lee KM, Yang SH, Choi S, Chung SY, et al: Involvement of hydrogen peroxide in mistletoe lectin-II-induced apoptosis of myeloleukemic U937 cells. Life Sci 2003;73:1231–1243.

17 Kim BK, Choi MJ, Park KY, Cho EJ: Protective effects of Korean mistletoe lectin on radical-induced oxidative stress. Biol Pharm Bull 2010;33:1152–1158.

18 Deliorman D, Calis I, Ergun F: A new acyclic monoterpene glucoside from *Viscum album* ssp. *album*. Fitoterapia 2001;72:101–105.

19 Deliorman D, Ergun F, Sener B, Palittapongarnpim P: Evaluation of antibacterial activity of *Viscum album* subspecies. Pharm Biol 2001;39:381–385.

20 Büssing A, Schietzel M: Apoptosis-inducing properties of *Viscum album* L. extracts from different host trees, correlate with their content of toxic mistletoe lectins. Anticancer Res 1999;19:23–28.

21 Radenkovic M, Ivetic V, Popovic M, Mimica-Dukic N, Veljkovic S: Neurophysiological effects of mistletoe (*Viscum album* L.) on isolated rat intestines. Phytother Res 2006;20:374–377.

22 Wollenweber E, Wieland A, Haas K: Epicuticular waxes and flavonol aglycones of the European mistletoe, *Viscum album* L. Z Nat Forsch 2000;55:314–317.

23 Maier G, Fiebig HH: Absence of tumor growth stimulation in a panel of 16 human tumor cell lines by mistletoe extracts in vitro. Anticancer Drugs 2002;13:373–379.

24 Kovacs E, Hajto T, Hostanska K: Improvement of DNA repair in lymphocytes of breast cancer patients treated with *Viscum album* extract (Iscador). Eur J Cancer 1991;27:1672.

25 Bussing A, Regnery A, Schweizer K: Effects of *Viscum album* L. on cyclophosphamide-treated peripheral blood mononuclear cells in vitro: sister chromatid exchanges and activation/proliferation marker expression. Cancer Lett 1995;94:199.

26 Kovacs E: The in vitro effect of *Viscum album* (VA) extract on DNA repair of peripheral blood mononuclear cells (PBMC) in cancer patients. Phytother Res 2002; 16:143–147.

27 Abraham P, Kolli VK, Rabi S: Melatonin attenuates methotrexate-induced oxidative stress and renal damage in rats. Cell Biochem Funct 2010;28:426–433.

28 Morsy MA, Ibrahim SA, Amin EF, Kamel MY, Rifaai RA, Hassan MK: Curcumin ameliorates methotrexate-induced nephrotoxicity in rats. Adv Pharmacol Sci 2013;2013:387071.

29 El-Sheikh AA, Morsy MA, Abdalla AM, Hamouda AH, Alhaider IA: Mechanisms of thymoquinone hepatorenal protection in methotrexate-induced toxicity in rats. Mediators Inflamm 2015;2015:859383.

30 Armagan I, Bayram D, Candan IA, Yigit A, Celik E, Armagan HH, et al: Effects of pentoxifylline and alpha lipoic acid on methotrexate-induced damage in liver and kidney of rats. Environ Toxicol Pharmacol 2015; 39:1122–1131.

31 Kim MH, Lee SH, Kim SC, Kim YK, Park SH: Comparative study on the effects of a *Viscum album* (L.) extract (mistletoe) and doxycycline for pleurodesis in patients with malignant pleural effusion. Korean J Med 1999;57(suppl 2):121.

32 Piao BK, Wang YX, Xie GR, Mansmann U, Matthes H, Beuth J, Lin HS: Impact of complementary mistletoe extract treatment on quality of life in breast, ovarian and non-small cell lung cancer patients. A prospective randomized controlled clinical trial. Anticancer Res 2004;24:303–309.

33 Reagan-Shaw S, Nihal M, Ahmad N: Dose translation from animal to human studies revisited. FASEB J 2008; 22:659–661.

34 Sekeroğlu V, Aydin B, Sekeroğlu ZA: *Viscum album* L. extract and quercetin reduce cyclophosphamide-induced cardiotoxicity, urotoxicity and genotoxicity in mice. Asian Pac J Cancer Prev 2011;12:2925–2931.

35 Abdel-Raheem IT, Khedr NF: Renoprotective effects of montelukast, a cysteinyl leukotriene receptor antagonist, against methotrexate-induced kidney damage in rats. Naunyn Schmiedebergs Arch Pharmacol 2014; 387:341–353.

36 Grönroos M, Chen M, Jahnukainen T, Capitanio A, Aizman RI, Celsi G: Methotrexate induces cell swelling and necrosis in renal tubular cells. Pediatr Blood Cancer 2006;46:624–629.

37 Chelab KG, Majeed SK: Methotrexate-induced histopathological changes in the kidneys of mice. Iraqi J Vet Sci 2009;23:219–222.

38 Çakır T, Polat C, Baştürk A, Gül M, Aslaner A, Durgut H, et al: The effect of alpha lipoic acid on rat kidneys in methotrexate induced oxidative injury. Eur Rev Med Pharmacol Sci 2015;19:2132–2139.

39 Ashtiyani SC, Najafi H, Firouzifar MR, Shafaat O: Grape seed extract for reduction of renal disturbances following reperfusion in rats. Iran J Kidney Dis 2013;7: 28–35.

40 Tenorio FA, del Valle L, González A, Pastelín G: Vasodilator activity of the aqueous extract of *Viscum album*. Fitoterapia 2005;76:204–209.

41 Tenorio-Lopez FA, Valle Mondragon LD, Olvera GZ, Torres Narvaez JC, Pastelin G: *Viscum album* aqueous extract induces NOS-2 and NOS-3 overexpression in Guinea pig hearts. Nat Prod Res 2006;20:1176–1182.

42 Lee JY, Kim JY, Lee YG, Byeon SE, Kim BH, Rhee MH, et al: In vitro immunoregulatory effects of Korean mistletoe lectin on functional activation of monocytic and macrophage-like cells. Biol Pharm Bull 2007;30:2043–2051.

43 Ibrahim MA, El-Sheikh AA, Khalaf HM, Abdelrahman AM: Protective effect of peroxisome proliferator activator receptor (PPAR)-α and -γ ligands against methotrexate-induced nephrotoxicity. Immunopharmacol Immunotoxicol 2014;36:130–137.

44 Kohnen S, Franck T, Van Antwerpen P, Boudjeltia KZ, Mouithys-Mickalad A, Deby C, et al: Resveratrol inhibits the activity of equine neutrophil myeloperoxidase by a direct interaction with the enzyme. J Agric Food Chem 2007;55:8080–8087.

45 Jahovic N, Cevik H, Sehirli AO, Yeğen BC, Sener G: Melatonin prevents methotrexate-induced hepatorenal oxidative injury in rats. J Pineal Res 2003;34:282–287.

46 Mollace V, Salvemini D, Anggard E, Vane J: Nitric oxide from vascular smooth cells: regulation of platelet reactivity and smooth muscle cell guanylate cyclase. Br J Pharmacol 1991;104:633–638.

47 Reiter RJ, Poeggeler B, Tan DX, Chen LD, Manchester LC, Guerrero JM: Antioxidant capacity of melatonin: a novel action not requiring a receptor. Neuroendocrinol Lett 1993;15:103–116.

48 Sekeroğlu ZA, Sekeroğlu V: Effects of *Viscum album* L. extract and quercetin on methotrexate-induced cytogenotoxicity in mouse bone-marrow cells. Mutat Res 2012;746:56–59.

49 Amic D, Davidovic-Amic D, Beslo D, Trinasjstic N: Structure-radical scavenging activity relationship of flavonoids. Croatia Chem Acta 2003;76:55–61.

50 Oboh G, Rocha JBT: Polyphenols in red pepper [*Capsicum annuum* var. *aviculare* (Tepin)] and their protective effect on some pro-oxidants induced lipid peroxidation in brain and liver. Eur Food Res Technol 2007; 225:239–247.

51 Ademiluyi AO, Oboh G: Antioxidant properties of methanolic extracts of mistletoes (*Viscum album*) from cocoa and cashew trees in Nigeria. Afr J Biotechnol 2008;7:3138–3142.

52 Shi ZM, Feng P, Jiang DQ, Wang XJ: Mistletoe alkali inhibits peroxidation in rat liver and kidney. World J Gastroenterol 2006;12:4052–4055.

53 Patrick-Iwuanyanwu KC, Onyeike EN, Wegwu MO: Anti-inflammatory effect of crude methanolic extract and fractions of African mistletoe *Tapinanthus bangwensis* (Engl. & K. Krause) on Wistar albino rats. Der Pharmacia Lettre 2010;2:76–83.

54 Dadhania VP, Tripathi DN, Vikram A, Ramarao P, Jena GB: Intervention of alpha-lipoic acid ameliorates methotrexate-induced oxidative stress and genotoxicity: a study in rat intestine. Chem Biol Interact 2010; 183:85–97.