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Protective Effect of Black Mulberry (*Morus nigra*) Fruit Extract on Methotrexate-related Intestinal Damage in Rats

Anna Carina ERGANİ[1], Ayşen TÜREDİ YILDIRIM[2], Fatma FIRAT[3], Seda VATANSEVER[4], İsmail TAŞTAN[5], Fatma TANELİ[6], Hüseyin GÜLEN[2]

[1]Konya City Hospital, Clinic of Pediatric Gastroenterology, Konya, Türkiye
[2]Manisa Celal Bayar University Faculty of Medicine, Department of Pediatric Hematology, Manisa, Türkiye
[3]Afyonkarahisar Health Sciences University Faculty of Medicine, Department of Histology and Embryology, Afyonkarahisar, Türkiye
[4]Manisa Celal Bayar University Faculty of Medicine, Department of Medicine & Department of Histology and Embryology, Manisa, Türkiye
[5]Akhisar Mustafa Kirazoğlu State Hospital, Manisa, Türkiye
[6]American Association for Clinical Chemistry, Celal Bayar University Faculty of Medicine, Department of Clinical Biochemistry, Manisa, Türkiye

ABSTRACT

Objective: In this study, we aimed to evaluate the potential protective effects of black mulberry (*Morus nigra*, MN) extract, known for its antioxidant properties, against gastrointestinal mucositis induced by high-dose methotrexate (MTX) treatment, assessed using histological and biochemical parameters.

Material and Methods: Male Wistar albino rats (200-250 g) were randomly assigned to four groups of 14 animals each:

- Group 1 received 30 mg/kg MTX intraperitoneally on day 1 to induce intestinal damage.
- Group 2 received 30 mg/kg MTX intraperitoneally on day 1, followed by 500 mg/kg of MN extract administered by gavage. One subgroup received MN for 4 days; the other subgroup received MN for 6 days.
- Group 3 received intragastric saline only (0.001 mL/kg-2.5 mL/kg).
- Group 4 received 500 mg/kg MN extract by oral gavage for 4 or 6 days, depending on the subgroup.

The animals were sacrificed on either day 4 or day 6. Blood samples were analyzed for total oxidant status (TOS) and total antioxidant status (TAS). Tissue samples from the duodenum, jejunum, and ileum were examined for levels of Ki-67, myeloperoxidase (MPO), malon dialdehyde (MDA), tumor necrosis factor-alpha (TNF-α), interleukin- 1 beta (IL-1β), TOS, and TAS.

Results: In the group treated with both MTX and MN extract, levels of MDA, MPO, IL-1β, and TNF-α were significantly lower than those in the MTX-only group. Ki-67 levels were higher in intestinal tissues, indicating enhanced epithelial regeneration.

Conclusion: Our findings suggest that MN extract protects against MTX-induced mucosal damage and promotes intestinal epithelial healing. These promising results highlight the potential of MN as a supportive treatment in preventing MTX-related toxicity; however, further large-scale and clinical studies are needed to confirm its efficacy.

Keywords: Childhood cancers; intestinal mucositis; methotrexate; *Morus nigra*; rat

INTRODUCTION

Despite the significant increase in life expectancy among cancer patients in recent years, attributable in part to the advent of effective treatment approaches, nutritional problems resulting from oral and gastrointestinal mucositis, which develop during treatment, remain a major cause of morbidity and mortality.[1] Approximately 400,000 new oral mucositis are diagnosed each year as a result of chemotherapy

Correspondence: Anna Carina ERGANİ MD;
Konya City Hospital, Clinic of Pediatric Gastroenterology, Konya, Türkiye
E-mail: drannaergani@gmail.com
ORCID ID: orcid.org/0000-0003-3494-1581

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(CT) and radiotherapy.[2] Methotrexate (MTX), a folic acid antimetabolite and antineoplastic chemotherapeutic agent, is widely used in the treatment of leukemia and various solid tumors. MTX can cause diarrhea, anorexia, malabsorption, malnutrition, and dehydration in patients. It also inhibits epithelial proliferation and enterocyte function, disrupting the intestinal mucosal barrier and causing mucositis. MTX-induced mucositis occurs on average between days 3 and 7 before the development of neutropenia and thrombocytopenia.[3] Intestinal mucositis is the most common cause of drug-related dose reductions, leading to malnutrition, growth retardation, and impaired quality of life.[4,5] Mucositis is not only an epithelial change but also a process in which reactive oxygen radicals, transcription factor activation, apoptosis, and cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and IL-6 are increased.[5]

New antioxidant agents are currently being tested to assess their efficacy in preventing or reducing CT-associated mucositis, a form of systemic oxidative damage. Antioxidant molecules, including but not limited to n-acetylcysteine, glutamine, L-carnitine, taurine, and melatonin, have demonstrated significant potential in reducing oxidative damage to the small intestine, liver, and kidneys.[6-9] Many agents are being investigated in both animal and clinical studies for the prevention and treatment of mucositis.

Mulberry is a fruit species that grows in temperate, tropical, and subtropical climates. *Morus alba* (white mulberry), *Morus nigra* (MN) (black mulberry), and *Morus rubra* (purple mulberry) are the most common species.[10] Mulberries have been demonstrated to be a source of numerous natural antioxidants, including flavonoids, phenolic acids, and vitamins C and E. Of these, mulberry phenolics are best known for their antioxidant properties.[11,12]

In this study, we investigated whether MN, which is known to have antioxidant properties, exerts beneficial effects on histopathological alterations and plasma oxidative stress parameters in MTX-induced mucositis of the rat small intestine. Biomarkers such as TNF-α, IL-1β, Ki-67, malon dialdehyde (MDA), and myeloperoxidase (MPO) were assessed immunohistochemically to evaluate the effect of MN. This experimental study is the first to investigate the effect of MN on MTX-induced oxidative damage.

MATERIAL AND METHODS

The present study was conducted on a group of 56 adult male Wistar albino rats, with a mean weight of 250-300 grams, which were housed in appropriate cages at a temperature of 24±1 °C on a 12 hours light/dark cycle and fed a standard laboratory diet. The experimental animals were divided into four groups, of 14 animals each. The subjects were divided into two subgroups, corresponding to fourth-day and sixth-day treatments, with seven subjects in each subgroup.

The animals used in this study were obtained from the Manisa Celal Bayar University Experimental Animals Application and Research Center. The Manisa Celal Bayar University Local Ethics Committee for Animal Experiments granted ethical approval (approval number: 77.637.435-64, date: 08.11.2016). Since this is an animal experiment, consent is not required.

The first group received a single intraperitoneal administration of 30 mg/kg MTX (Koçak Farma Pharmaceuticals, İstanbul, Türkiye) on the first day. The second group received a single intraperitoneal dose of 30 mg/kg MTX on the first day, followed by oral gavage of 500 mg/kg MN extract commencing after the MTX administration and continuing for four days in the first subgroup and six days in the second subgroup. The third group received an intragastric saline solution at doses ranging from 0.001 mL/kg to 2.5 mL/kg for four days in the first subgroup and six days in the second subgroup (control group). The fourth group was given an oral gavage of MN extract at a dose of 500 mg/kg for four days in the first subgroup and six days in the second subgroup (Table 1). The subjects were sacrificed under general anesthesia on either the fourth or sixth day, depending on the study group, and approximately 3 mL of cardiac blood was collected from each animal under sterile conditions. Intestinal tissues were also removed.

Paraffin Tissue Tracking

After fixation, all samples were kept under running water overnight. They were then kept in 60%, 70%, and 80% alcohol solutions for 30 minutes each, and dehydrated in

TABLE 1: Formation of the experimental groups.

Groups	Application	Day 4	Medicine day	Day 6	Medicine day
Group 1	30 mg/kg MTX	7	1 day	7	1 day
Group 2	30 mg/kg MTX + 500mg/kg MN extract	7	1 day MTX + 4 day MN	7	1 day MTX + 6 days MN
Group 3	0.001 mL/kg-2.5 mL/kg intragastric saline solution (control)	7	4 days	7	6 days
Group 4	500mg/kg MN extract	7	4 days	7	6 days

MTX: Methotrexate; MN: *Morus nigra*.

100% alcohol for 1 hour with two changes. After being kept in xylene-alcohol solution for 30 minutes, they were transferred to xylene and kept for 1 hour with two changes for transparency. All samples were kept in an xylene-paraffin mixture for 30 minutes and embedded in paraffin.

Histochemical Staining

The samples taken from the animals were sectioned at 5 microns and kept overnight at 60 °C for paraffinization. The next day, the sections were placed in xylene for 1 hour, washed in running water for 5 minutes, and then transferred through a series of alcohols (96%, 80%, 70%, and 60%) for 2 minutes each. The sections were washed again for 5 minutes and kept in hematoxylin (Haematoxylin Harris, RRSP67/E) for 3 minutes. Then they were washed in running water for 5 minutes, immersed in 1% acid-alcohol solution, and removed. It was washed again in running water for 5 minutes and stained with eosin (Surgipath, Unno1989, 01601E) for 1 minute. After washing in running water for 5 minutes, the sections were soaked in 80% and 96% alcohol, each for 1 minute, then dried and immersed in xylene for 1 hour. All stained slides were covered with Entellan (Spring Bioscience, DMM-125) and examined under a light microscope(Olympus, BX43).

Immunohistochemistry

For immunohistochemical staining, the sections were incubated in an oven at 60 °C overnight, followed by clearing in two changes of xylene, 30 minutes each. They were then rehydrated through a graded series of alcohols ranging from 95% to 60% and then kept in distilled water for 10 minutes. The samples were outlined with an immunohistochemistry pen (IM3580, Immunotech, Marseille, France) and incubated in 0.5% trypsin solution (EK001-10K, Biogenex, San Ramon, USA) for 15 min at room temperature. Sections were washed three times for 5 minutes each with phosphate buffer (PBS). Three percent H_2O_2 was applied for 5 minutes to inhibit endogenous tissue peroxidase. The sections were washed with PBS 3 times for 5 minutes each and then treated with blocking solution (85-9043, Histostainplus kit, Invitrogen) for 10 minutes. After the blocking solution was removed from the tissue, the sections were incubated overnight with primary antibodies at a 1/100 dilution: anti-MPO (D3ZGE2), anti-Ki-67 (ABM40064, Abbkine), anti-TNF-α (ABP52624, Abbkine), anti-IL-1β (Abp51611, Abbkine), and anti-MDA (ab6463, Abcam). The next day, the sections were washed 3 times with PBS and stained with anti-mouse/anti-rabbit biotin-streptavidin hydrogen peroxidase secondary antibody (85-9043, Histostainplus kit, Invitrogen) for 30 minutes per staining step. The sections were washed three times with PBS for 5 minutes each between steps, and stained with diaminobenzidine (catalog nos. 71897 and 71898; Millipore) for 5 minutes to visualize the immunohistochemical reaction. After background staining with Mayer's hematoxylin (02274390059, J.T.Barker, Deventer, Holland) for 2 minutes, the sections were washed with distilled water for 10 minutes and then covered with a closing medium (K002, DBS, California, USA).

Immunohistochemical Assessments

Histochemical and immunohistochemical staining (for MPO, MDA, IL-1β, Ki-67, and TNF-α antibodies) was performed on the small intestine. A semiquantitative method was used for immunohistochemical evaluation; the histologic score (H-score) was determined based on the distribution area and staining intensity of the cells. Histopathological and immunohistochemical evaluations were performed by investigators blinded to group allocation. The mean proportion of stained cells was graded as follows: 0 for <1% of the stained area, 1 for 1% to 25%, 2 for 26% to 50%, 3 for 51% to 75%, and 4 for >75%. Staining intensity was graded as follows: 0, negative staining; 1, weak staining; 2, moderate staining; 3, strong staining. The H-score for each specimen was calculated as follows:

H-score = degree of cell area-stained X average staining intensity

A total score of 0 to 12 was calculated and rated as negative (-, score: 0), weak (+, score: 1 to 4), moderate (++, score: 5 to 8) or strong (++++, score: 9 to 12).[13]

Biochemical Analysis

Blood samples from the rats were collected intracardially into lithium-heparinized tubes. Blood samples were centrifuged at 3500 rpm for 10 minutes, and plasma was separated and stored at -80 °C until analyzed in bulk. Plasma samples were analyzed for total oxidant status (TOS) and total antioxidant status (TAS) using a spectrophotometric method with commercial kits (RelAssay, Gaziantep, Türkiye). In the TOS analysis, total antioxidant molecules in the sample were measured spectrophotometrically, and the kit was calibrated using hydrogen peroxide. Results were given as micromol hydrogen peroxide equivalent/liter (μmol H_2O_2 Eq/L).[14] TAS was analyzed via the antioxidant effect of ABST (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation, and the results were given as micromol Trolox equivalent/L.[15] The oxidative stress index (OSI) is an indicator of oxidative stress.

The TOS/TAS ratio is calculated using the following formula.[16]

OSI (arbitraryunit) = [TOS (μmol H_2O_2 Eq/L)/TAS (μmol Trolox Eq/L)] × 100.

Obtaining *Morus nigra*

At the Ege University Faculty of Pharmacy, Department of Pharmacognosy Research Laboratory, the fruits were brought to room temperature and homogenized using a blender. They were macerated in 70% aqueous ethanol (10k to 1k fruit homogenate) for 24 hours on a shaker, and then filtered through filter paper. The filtrate was evaporated to dryness under reduced pressure using a rotary evaporator. The extract was stored in the refrigerator.

Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics for Windows, version 26.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics were expressed as mean ± standard deviation for continuous variables and as number (n) and percentage (%) for categorical variables. The normality of data distribution was assessed using the Shapiro-Wilk test. For comparisons involving multiple groups and time points (days 4 and 6), a two-way mixed-design analysis of variance was applied to evaluate the effects of treatment groups, time, and their interaction. When significant differences were detected, post hoc pairwise comparisons were performed using the Bonferroni correction method. For non-normally distributed data, appropriate non-parametric tests were applied (Kruskal-Wallis test for multiple group comparisons and Mann-Whitney U test for pairwise comparisons). Categorical variables were compared using the chi-square test where applicable. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Macroscopic Findings

All subjects tolerated MTX. After sacrifice, intestinal tissues were removed, and macroscopic observations were made. In groups 1 and 2, swelling (edema) and mucositis were observed in the intestine and anus; foci of hyperemic hemorrhage were observed in Group 1. In Groups 3 and 4, no macroscopic changes were observed in any segment of the intestine (Figure 1). In addition, no pathologically enlarged mesenteric lymph nodes were found in any of the four groups.

Light Microscopic Findings

Light-microscopic examination revealed epithelial irregularities, polymorphonuclear leukocytes (PNL), and bleeding foci in the lamina propria of samples from the duodenum, jejunum, and ileum in Group 1. In Group 2, epithelial irregularities decreased; PNL infiltration was observed, but erythrocytes were absent from the lamina propria. Single-layered prismatic epithelium, loose connective tissue, and a normal lamina propria were observed in all intestinal samples from Groups 3 and 4. No histologic changes were observed in the muscular and adventitial layers in any of the intestinal specimens examined.

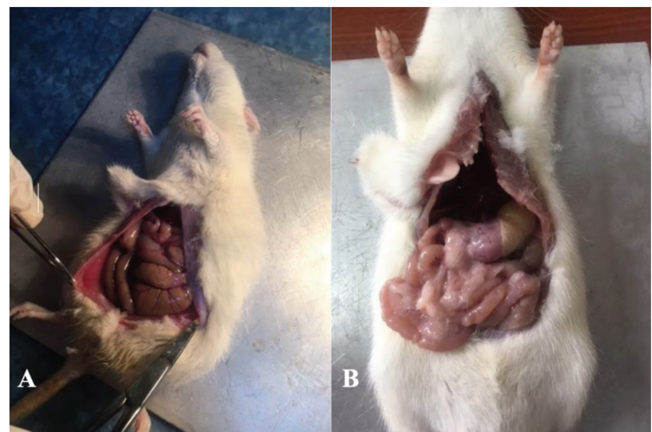

FIGURE 1: Macroscopic appearance of intestinal tissues after sacrifice (**A**: Normal intestinal tissue; **B**: congested and edematous intestinal wall).

Immunohistochemical Findings

Immunohistochemical Evaluations

IL-1β, Ki-67, TNF-α, MDA, and MPO were analyzed immunohistochemically in samples from the duodenum, jejunum, and ileum. A semiquantitative method was used for immunohistochemical evaluation, and the H-Score was determined based on the distribution area and staining intensity of the stained cells.

IL-1β

Analysis of Table 2 showed that IL-1β measurements taken on days 4 and 6 exhibited statistically significant differences between groups and between regions. On day 4, IL-1β measurements from the ileum region in the MTX + MN group were statistically higher than those from the duodenum and jejunum regions ($p<0.05$). On day 6, in the MTX + MN group, IL-1β measurements from the duodenum were higher than those from other regions, while measurements from the jejunum were significantly lower than those from other regions ($p<0.05$). On days 4 and 6, IL-1β measurements in the MTX group were significantly higher than those in the other groups across all regions ($p<0.05$). IL-1β measurements in the duodenum and jejunum of the MTX + MN group on day 4 were significantly lower than the measurements taken on day 6 ($p<0.05$). The IL-1β measurements taken on day 4 in the jejunum of the MTX group, the ileum of the MTX + MN group, and the duodenum of the MN group were significantly higher than the measurements taken on day 6 ($p<0.05$) (Table 2, Figure 2).

TABLE 2: Immunohistochemical evaluation of IL-1β measurement between regions and groups.				
Group	Regions	IL-1β		Test statistics$^{\phi}$
		Day 4	Day 6	
MTX	Duodenum	11.25±1.39^{A}	10.88±1.55^{A}	p=0.598, η^2=0.04
	Jejunum	11.63±1.06^{A}	8.75±0.46^{B}	**p<0.001, η^2=0.93**
	Ileum	11.25±1.39^{A}	11.63±1.06^{A}	p=0.351, η^2=0.13
MTX+MN	Duodenum	3.38±0.52^{D}	7.00±1.85^{C}	**p<0.001, η^2=0.84**
	Jejunum	3.88±0.35^{D}	2.13±0.35^{E}	**p<0.001, η^2=0.94**
	Ileum	8.50±0.53^{B}	4.75±1.04^{D}	**p<0.001, η^2=0.88**
Control	Duodenum	1.00±0.53^{E}	0.88±0.35^{E}	p=0.685, η^2=0.03
	Jejunum	0.50±0.53^{E}	0.63±0.52^{E}	p=0.598, η^2=0.04
	Ileum	1.50±0.53^{E}	1.25±0.46^{E}	p=0.351, η^2=0.13
MN	Duodenum	3.75±0.46^{D}	2.00±0.53^{E}	**p<0.001, η^2=0.82**
	Jejunum	1.00±0.00^{E}	1.00±0.00^{E}	p=0.999, η^2=0.00
	Ileum	1.13±0.35^{E}	1.25±0.46^{E}	p=0.598, η^2=0.04
Test statistics†		**p<0.001, η^2=0.97**	**p<0.001, η^2=0.96**	

Effect size (η^2); $^{\phi}$: within-group comparison; †: between-group comparison. Data are presented as mean ± standard deviation ($\bar{X}$ ± SD). Bold values indicate statistical significance (p<0.05). Values with different superscript letters (A-E) within the same row or column differ significantly according to the Bonferroni post-hoc test (p<0.05).
IL: Interleukin; MTX: Methotrexate; MN: Morus nigra.

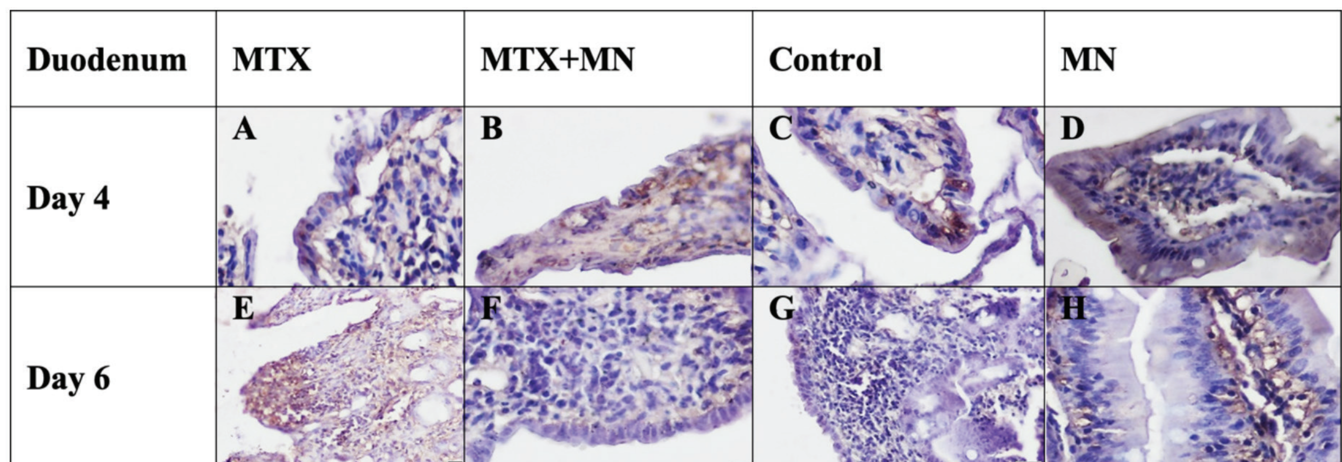

FIGURE 2: Representative immunohistochemical staining images of IL-1β in the duodenum in the MTX, MTX + MN, control, and MN groups on days 4 and 6. **(A-D)** day 4; **(E-H)** day 6. **(A, E)** MTX; **(B, F)** MTX + MN; **(C, G)** control; **(D, H)** MN groups. Images were captured at ×200 and ×400 magnifications (scale bars: 20 µm and 10 µm, respectively).

MTX: Methotrexate; MN: Morus nigra

Ki-67

Thorough examination of Table 3 revealed statistically significant differences in Ki-67 measurements on days 4 and 6 among groups and regions. On day 4, the ileum of the MTX + MN group exhibited significantly lower Ki-67 measurements than the duodenum and jejunum (p<0.05). On the fourth day, the Ki-67 measurements from the duodenum and jejunum in the MTX + MN group, and from the ileum in the Control and MN groups, were statistically higher than those in the other groups (p<0.05). On day 6, Ki-67 levels in the duodenum of the MTX + MN group were higher than in other regions, whereas levels in the ileum were statistically lower than in other regions (p<0.05).On day 6, the Ki-67 measurements in the duodenum and jejunum regions were significantly higher than those in the MTX + MN group (p<0.05). Furthermore, the Ki-67 measurements taken on day 4 in the jejunal regions of the MTX + MN group, in the ileal and jejunal regions of the

TABLE 3: Immunohistochemical evaluation of Ki-67 measurement between regions and groups.

Group	Regions	Ki-67		Test statistics[φ]
		Day 4	Day 6	
MTX	Duodenum	1.00±0.00[F]	1.13±0.35[E,F]	p=0.351, η^2=0.13
	Jejunum	0.63±0.52[F]	1.00±0.00[F]	p=0.080, η^2=0.38
	Ileum	1.00±0.00[F]	1.13±0.35[E,F]	p=0.351, η^2=0.13
MTX+MN	Duodenum	6.75±1.04[A]	7.00±1.07[A]	p=0.732, η^2=0.02
	Jejunum	6.00±0.00[A]	4.50±0.93[B]	**p=0.003, η^2=0.75**
	Ileum	1.75±0.46[D]	1.63±0.52[D]	p=0.598, η^2=0.04
Control	Duodenum	2.88±0.35[D]	2.75±0.46[D]	p=0.598, η^2=0.04
	Jejunum	2.25±0.46[D]	1.00±0.00[F]	**p<0.001, η^2=0.89**
	Ileum	3.63±0.52[C]	2.25±0.46[D]	**p=0.004, η^2=0.72**
MN	Duodenum	4.00±0.00[C]	3.00±0.00[D]	**p<0.001, η^2=0.99**
	Jejunum	1.00±0.00[F]	1.50±0.53[E]	**p=0.033, η^2=0.50**
	Ileum	3.63±0.52[C]	2.63±0.74[D]	**p=0.033, η^2=0.50**
Test statistics[†]		**p<0.001, η^2=0.95**	**p<0.001, η^2=0.91**	

Effect size (η^2); φ: within-group comparison; †: between-group comparison. Data are presented as mean ± standard deviation ($\bar{X}$ ± SD). Bold values indicate statistical significance (p<0.05). Values with different superscript letters (A-E) within the same row or column differ significantly according to the Bonferroni post-hoc test (p<0.05).
MTX: Methotrexate; MN: *Morus nigra*.

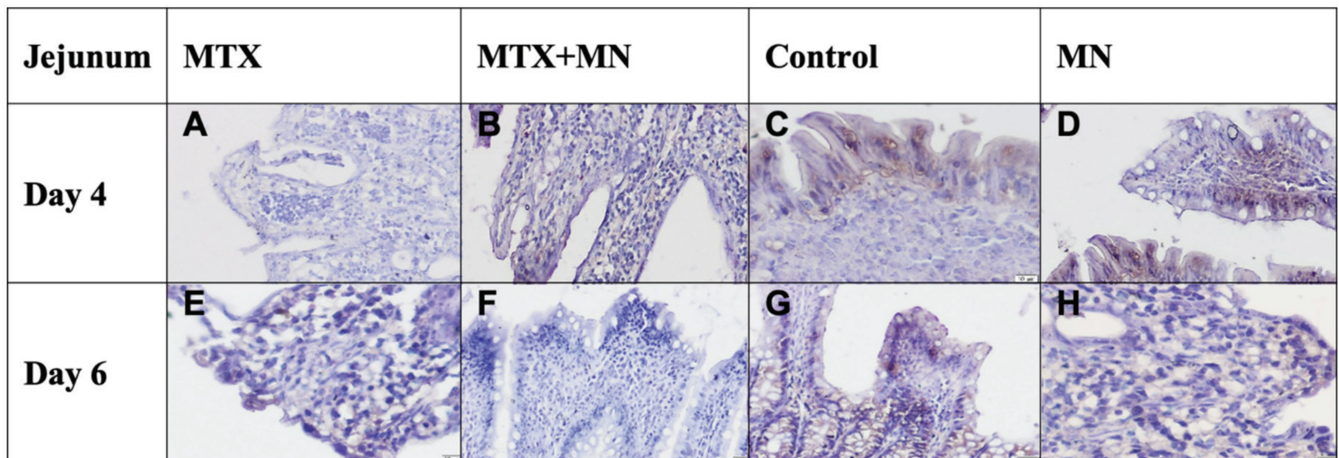

FIGURE 3: Representative immunohistochemical staining images of Ki-67 in the duodenum in the MTX, MTX + MN, control, and MN groups on days 4 and 6. **(A-D)** day 4; **(E-H)** day 6. **(A, E)** MTX; **(B, F)** MTX + MN; **(C, G)** control; **(D, H)** MN groups. Images were captured at ×200 and ×400 magnifications (scale bars: 20 µm and 10 µm, respectively).

MTX: Methotrexate; MN: Morus nigra

control group, and in the duodenal and ileal regions of the MN group were significantly higher than those taken on day 6 (p<0.05) (Table 3, Figure 3).

TNF-α

A subsequent analysis of Table 4 revealed statistically significant differences in TNF-α measurements among the groups and regions. On day 4, TNF-α levels in the ileum of the MTX + MN group were significantly higher than those in the duodenum and jejunum (p<0.05). Similarly, on day 4, the TNF-α measurement in the Mtx group was significantly higher compared with the other groups across all regions (p<0.05). On day 6, no statistically significant differences in TNF-α measurements were observed among the regions within the MTX + MN group (p>0.05). However, TNF-α measurements on day 4 in all regions of the MTX group and in the ileum region of the MTX + MN group were statistically higher than measurements taken on day 6 (p<0.05) (Table 4, Figure 4).

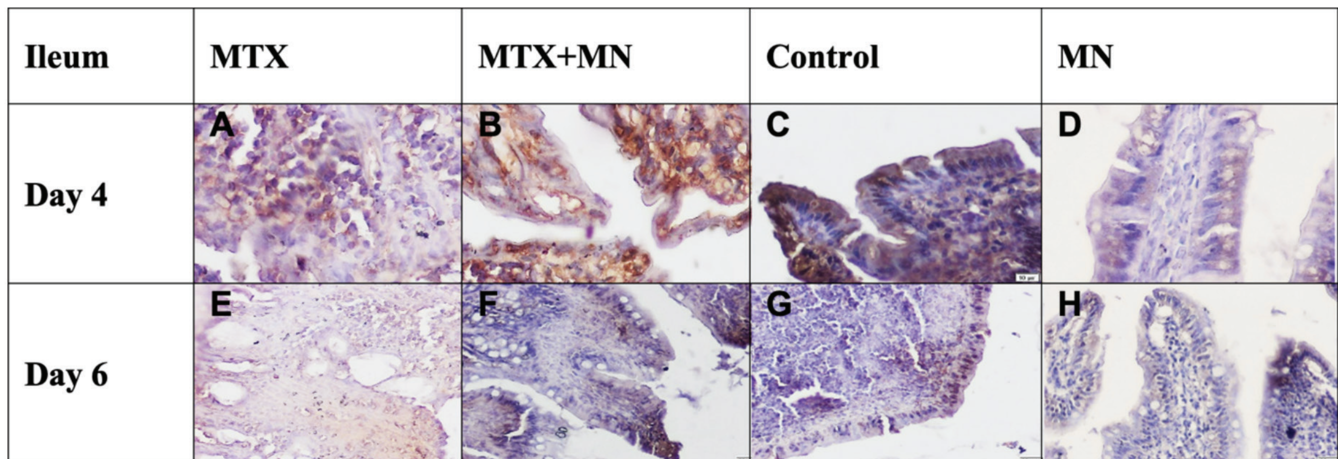

FIGURE 4: Representative immunohistochemical staining images of TNF-α in the duodenum in the MTX, MTX+MN, control, and MN groups on days 4 and 6. **(A-D)** day 4; **(E-H)** day 6. **(A, E)** MTX; **(B, F)** MTX + MN; **(C, G)** control; **(D, H)** MN groups. Images were captured at ×200 and ×400 magnifications (scale bars: 20 µm and 10 µm, respectively).

TNF-α: Tumor necrosis factor-alpha; MTX: Methotrexate; MN: Morus nigra

TABLE 4: Immunohistochemical evaluation of TNF-α measurement between regions and groups.

Group	Regions	TNF-α		Test statistics$^{\phi}$
		Day 4	Day 6	
MTX	Duodenum	8.75±0.46^{B}	4.50±0.93^{D}	**p<0.001, η^2=0.91**
	Jejunum	11.63±1.06^{A}	8.88±0.35^{B}	**p<0.001, η^2=0.86**
	Ileum	10.88±1.55^{A}	6.25±0.71^{C}	**p<0.001, η^2=0.92**
MTX+MN	Duodenum	3.25±0.46^{D}	3.25±0.46^{D}	p=0.999, η^2=0.00
	Jejunum	4.00±0.00^{D}	4.00±000^{D}	p=0.999, η^2=0.00
	Ileum	8.75±0.46^{B}	3.13±0.35^{D}	**p<0.001, η^2=0.99**
Control	Duodenum	3.38±1.06^{D}	3.25±0.46^{D}	p=0.785, η^2=0.01
	Jejunum	1.13±0.35^{E}	1.25±0.46^{E}	p=0.598, η^2=0.04
	Ileum	2.75±0.46^{D}	2.75±0.46^{D}	p=0.999, η^2=0.00
MN	Duodenum	4.25±0.71^{D}	5.75±0.71^{C}	**p=0.020, η^2=0.56**
	Jejunum	0.50±0.53^{E}	0.75±0.46^{E}	p=0.451, η^2=0.08
	Ileum	1.75±0.46^{E}	2.75±0.46^{D}	**p=0.007, η^2=0.67**
Test statistics†		**p<0.001, η^2=0.97**	**p<0.001, η^2=0.95**	

Effect size (η^2); φ: within-group comparison; †: between-group comparison. Data are presented as mean ± standard deviation (X̄ ± SD). Bold values indicate statistical significance (p<0.05). Values with different superscript letters (A-E) within the same row or column differ significantly according to the Bonferroni post-hoc test (p<0.05).
TNF-α: Tumor necrosis factor-alpha; MTX: Methotrexate; MN: *Morus nigra*.

MDA

Analysis of Table 5 revealed statistically significant differences in MDA measurements across groups and regions. On day 4, the MDA measurements from the jejunum region in the MTX + MN group were significantly higher than those in the duodenum and ileum regions (p<0.05). On Day 4, MDA measurements in the duodenum and ileum from the MTX group and in the jejunum from the MTX + MN group were statistically higher than those in the other groups (p<0.05). On day 6, the MDA measurement in the jejunum region in the MTX + MN group was significantly higher than in the duodenum and ileum regions (p<0.05). On day 6, the MDA measurement in the MTX group was statistically significant compared with the other groups across all regions (p<0.05). Furthermore, the MDA measurements obtained on day 4 from the MTX + MN group in all regions were significantly higher than those obtained on day 6 (p<0.05) (Table 5, Figure 5).

MPO

Analysis of Table 6 showed that MPO measurements taken on days 4 and 6 differed significantly across groups and regions.

TABLE 5: Immunohistochemical evaluation of MDA measurement between regions and groups.

Group	Regions	MDA		Test statistics$^{\phi}$
		Day 4	Day 6	
MTX	Duodenum	11.63±1.06^{A}	11.25±1.39^{A}	p=0.598, η^2=0.04
	Jejunum	9.00±0.00^{B}	9.00±0.00^{B}	p=0.999, η^2=0.00
	Ileum	11.63±1.06^{A}	11.25±1.39^{A}	p=0.598, η^2=0.04
MTX+MN	Duodenum	10.88±1.55^{B}	2.50±0.53^{D}	**p<0.001, η^2=0.96**
	Jejunum	12.00±0.00^{A}	9.00±0.00^{B}	**p<0.001, η^2=0.99**
	Ileum	8.63±0.52^{B}	5.50±0.93^{C}	**p<0.001, η^2=0.88**
Control	Duodenum	0.75±0.46^{E}	0.75±0.46^{E}	p=0.999, η^2=0.00
	Jejunum	1.00±0.00^{E}	1.00±0.00^{E}	p=0.999, η^2=0.00
	Ileum	2.13±0.64^{D}	2.13±0.83^{D}	p=0.999, η^2=0.00
MN	Duodenum	0.13±0.35^{E}	0.38±0.52^{E}	p=0.351, η^2=0.13
	Jejunum	2.00±0.00^{D}	2.00±0.00^{D}	p=0.999, η^2=0.00
	Ileum	2.25±0.71^{D}	2.13±0.35^{D}	p=0.685, η^2=0.02
Test statistics†		**p<0.001, η^2=0.98**	**p<0.001, η^2=0.97**	

Effect size (η^2); φ: within-group comparison; †: between-group comparison. Data are presented as mean ± standard deviation ($\bar{X}$ ± SD). Bold values indicate statistical significance (p<0.05). Values with different superscript letters (A-E) within the same row or column differ significantly according to the Bonferroni post-hoc test (p<0.05).
MDA: Malon dialdehyde; MTX: Methotrexate; MN: *Morus nigra*.

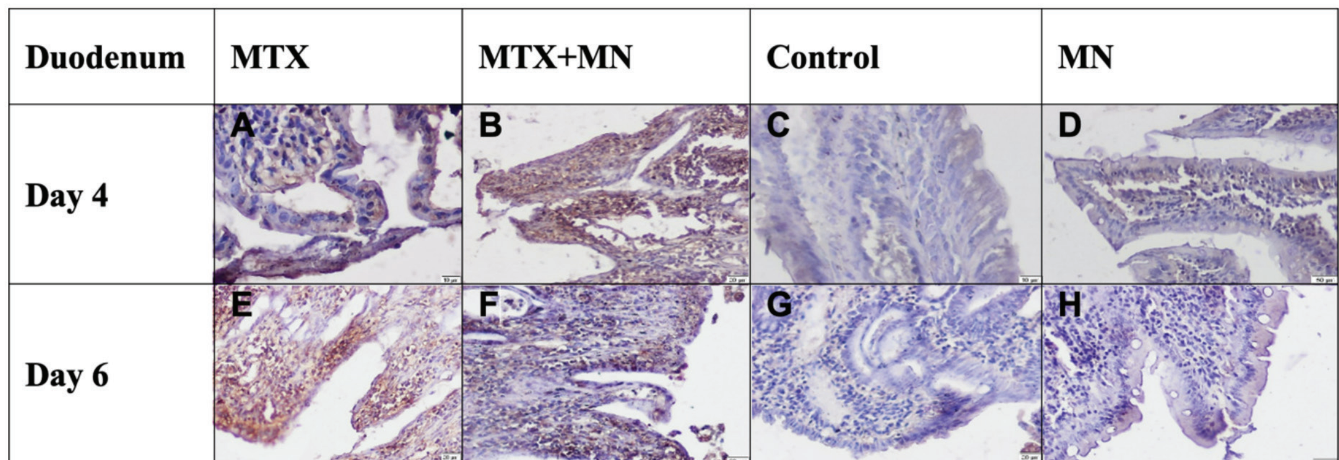

FIGURE 5: Representative immunohistochemical staining images of MDA in the duodenum in the MTX, MTX + MN, control, and MN groups on days 4 and 6. **(A-D)** day 4; **(E-H)** day 6. **(A, E)** MTX; **(B, F)** MTX + MN; **(C, G)** control; **(D, H)** MN groups. Images were captured at ×200 and ×400 magnifications (scale bars: 20 µm and 10 µm, respectively).

MDA: Malon dialdehyde; MTX: Methotrexate; MN: Morus nigra

On Day 4, MPO measurement in the ileum region in the MTX + MN group was significantly higher than measurements in the duodenum and jejunum regions (p<0.05). On days 4 and 6, MPO measurements in the MTX group were significantly higher than those in the other groups across all regions (p<0.05) (Table 6, Figure 6).

Biochemical Findings

Evaluation of the TAS on day 6 revealed that the difference between Group 1 and Group 2 was not statistically significant (p=1.000). However, the increase in TAS value was statistically significant compared with Groups 3 and 4 (p=0.020, p=0.003, respectively). Compared with Group 2, Group 4 showed a statistically significant increase in TAS value (p=0.010). No statistically significant differences were observed between the groups on days 4 and 6 for TOS values (Table 7).

DISCUSSION

This study investigated the protective effect of MN on MTX-induced small intestinal mucositis in rats. Our

TABLE 6: Immunohistochemical evaluation of MPO measurement between regions and groups.

Group	Regions	MPO		Test statistics$^{\varphi}$
		Day 4	Day 6	
MTX	Duodenum	11.63±1.06^{A}	11.25±1.39^{A}	p=0.598, η^2=0.04
	Jejunum	12.00±0.00^{A}	12.00±0.00^{A}	p=0.999, η^2=0.00
	Ileum	8.75±0.46^{B}	8.88±0.35^{B}	p=0.598, η^2=0.04
MTX+MN	Duodenum	1.13±0.35^{E}	1.25±0.46^{E}	p=0.598, η^2=0.04
	Jejunum	2.25±0.46^{D}	2.13±0.35^{D}	p=0.598, η^2=0.04
	Ileum	5.75±0.71^{C}	5.50±0.93^{C}	p=0.598, η^2=0.04
Control	Duodenum	0.50±0.53^{E}	0.38±0.52^{E}	p=0.685, η^2=0.03
	Jejunum	1.75±0.46^{D}	1.75±0.46^{D}	p=0.999, η^2=0.00
	Ileum	2.25±0.46^{D}	2.25±0.46^{D}	p=0.999, η^2=0.00
MN	Duodenum	2.00±0.53^{D}	1.88±0.83^{D}	p=0.763, η^2=0.01
	Jejunum	1.75±0.46^{D}	1.63±0.52^{D}	p=0.685, η^2=0.03
	Ileum	2.13±0.99^{D}	2.25±0.46^{D}	p=0.763, η^2=0.01
Test statistics†		**p<0.001, η^2=0.98**	**p<0.001, η^2=0.98**	

Effect size (η^2); φ: within-group comparison; †: between-group comparison. Data are presented as mean ± standard deviation ($\bar{X}$ ± SD). Bold values indicate statistical significance (p<0.05). Values with different superscript letters (A-E) within the same row or column differ significantly according to the Bonferroni post-hoc test (p<0.05).
MPO: Myeloperoxidase; MTX: Methotrexate; MN: *Morus nigra*.

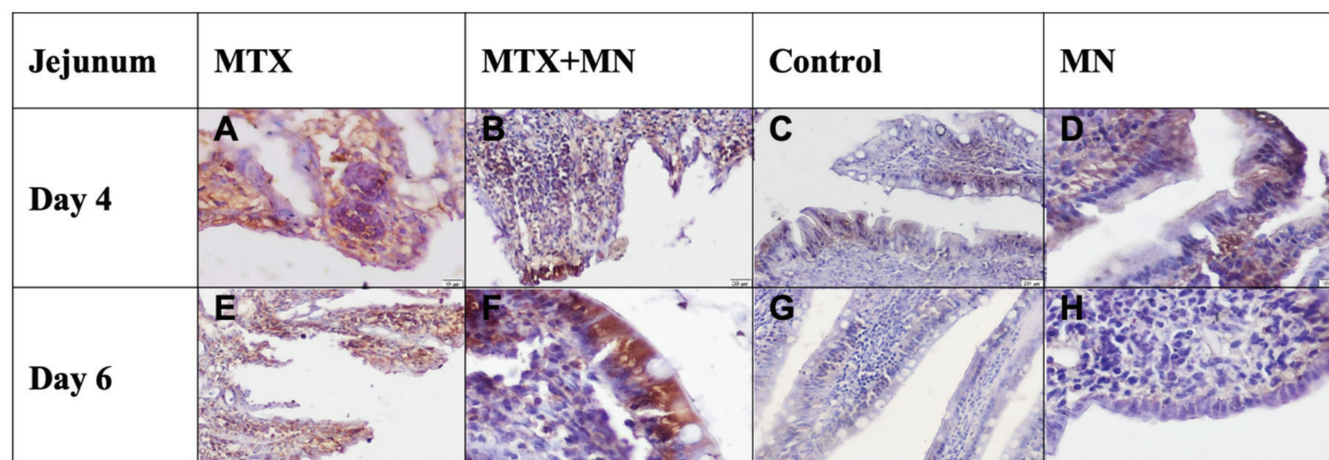

FIGURE 6: Representative immunohistochemical staining images of MPO in the duodenum in the MTX, MTX + MN, control, and MN groups on days 4 and 6. **(A-D)** day 4; **(E-H)** day 6. **(A, E)** MTX; **(B, F)** MTX + MN; **(C, G)** Control; **(D, H)** MN groups. Images were captured at ×200 and ×400 magnifications (scale bars: 20 µm and 10 µm, respectively).

MPO: Myeloperoxidase; MTX: Methotrexate; MN: Morus nigra

immunohistochemical results showed that MTX increased the levels of oxidative stress markers (MDA and MPO) and inflammatory markers (IL-1β and TNF-α), decreased the level of Ki-67, a proliferation marker, in the duodenum, jejunum, and ileum of rats. When we compared the rats given MTX and 500 mg/kg MN extract together with the rats given MTX alone, MDA, MPO, IL-1β, and TNF-α levels decreased in the MN-treated group, while Ki-67 levels increased in small intestinal tissues. Histopathological examination showed that 500 mg/kg MN extract could provide adequate protection of small intestinal tissue against MTX-induced oxidative damage. Our biochemical results showed that MN had no TAS-enhancing effect.

MTX induced epithelial disorganization, PNL-cell infiltration, and bleeding foci in the small intestinal tissues of rats. Histopathologic examination after the addition of MN showed that MTX-induced epithelial disorganization and intestinal mucositis had resolved. Proliferation and self-renewal of small intestinal epithelial cells occur in crypts. Crypt cells, which can regenerate very rapidly under normal physiologic conditions, migrate to the ends of the villi and enable the gastrointestinal

TABLE 7: Statistical data of total antioxidant status (TAS) and total oxidant status (TOS) values.				
Group	TAS (1.2-1.5 milimol/L)		TOS (4-6 micromol/L)	
	Day 4	Day 6	Day 4	Day 6
	Average ± SD	Median (min/max)	Median (min/max)	Average ± SD
1	1.88±0.50	1.10 (0.95/1.42)	98.43 (39.13/221.69)	34.08±11.42
2	1.52±0.22	1.17 (1.05/1.25)	46.87 (22.58/158.31)	31.48±12.60
3	1.61±0.10	1.57 (1.46/2.10)	41.63 (16.92/104.79)	35.77±18.87
4	1.46±0.24	1.68 (1.35/1.79)	30.77 (23.58/86.16)	42.17±24.79
p-value	0.093	**<0.001**	0.110	0.697
1→2	ad.	1.000	ad.	ad.
1→3	ad.	**0.020**	ad.	ad.
1→4	ad.	**0.003**	ad.	ad.
2→3	ad.	0.053	ad.	ad.
2→4	ad.	**0.010**	ad.	ad.
3→4	ad.	1.000	ad.	ad.

OneWay ANOVA (Brown-Forsythe), Kruskal-Wallis test (Monte Carlo); Post-hoc test: Dunn's Test.
ANOVA: Analysis of variance; SD: Standard deviation; Min: Minimum; Max: Maximum; TAS: Total antioxidant status; TOS: Total oxidative status; OSI: Oxidative stress index; 1st group: Methotrexate; 2nd group: Methotrexate + *Morus nigra*; 3rd group: Control; 4th group: *Morus nigra*.

epithelium to renew itself completely in approximately 2-3 days. Miyazono et al.[17] showed that oxidative stress, especially neutrophil infiltration, plays a role in damage after MTX treatment. Taminiau et al.[18] have shown that the most severe effects of MTX-induced histopathologic changes are crypt and villous ablation in the proximal small intestine. For this reason, we examined all parts of the small intestine. We observed that macroscopic changes and mucositis in the small intestine of sacrificed rats decreased from proximal to distal regions. The jejunal epithelium enters a highly proliferative phase 96 hours after MTX administration. Increased mitotic activity, deepened crypts, and crypt hyperplasia have been shown to be greatest on days 4 to 6 after MTX administration.[19] Therefore, we collected duodenal, jejunal, and ileal tissues under deep anesthesia at 96 and 144 hours after a single intraperitoneal dose of MTX for histopathologic evaluation of small intestinal injury. Immunohistochemically, MTX-induced intestinal injury was observed on days 4 and 6. At the end of our study, immunohistochemical scores for oxidant and inflammatory parameters in the small intestine were lower in the MTX + MN group, whereas proliferative scores were higher in the MTX group. This finding suggests that the addition of MN to MTX reduces intestinal damage.

Previous studies have confirmed that MTX toxicity is associated with increased inflammatory cytokine production.[20,21] Antioxidant activity and inhibition of IL-1β may help prevent MTX-induced intestinal inflammation.[22] de Araújo et al.[23] reported that in addition to oxidants, proinflammatory IL-1β and TNF-α levels were significantly increased in damaged intestinal tissue. In our study, we showed that the levels of the proinflammatory cytokines IL-1β and TNF-α and the oxidant/antioxidant balance in small intestinal tissues shifted in favor of oxidants in Group 1. However, in Group 2, MN suppressed the increase in IL-1β and TNF-α levels in small intestinal tissues by preventing a shift in the balance toward oxidants. Although MTX increased IL-1β and TNF-α levels, simultaneous administration of MN prevented MTX-induced deleterious effects in all small intestinal segments. According to our results, MN showed anti-inflammatory properties by decreasing IL-1β and TNF-α secretion.

Sugiyama et al.[24] reported that the rate of Ki-67 antigen-positive cells decreased significantly in those given MTX. In a similar study, Leitão et al.[25] found that the number of Ki-67 positive cells, a proliferation marker, decreased significantly on day 5 in the jejunum epithelium of rats given MTX. The absence of Ki-67 immunoreactivity in Group 1 supports that MTX inhibits epithelial proliferation; the observation of increases in Ki-67, especially in the duodenum and jejunum, as a result of MN administration in Group 2, supports that the extract triggers proliferation to prevent epithelial damage.

Active oxygen species are known to play a role in MTX-induced oxidative stress.[26] Odabasoglu et al.[27] reported increased MDA and decreased total glutathione in inflammatory tissue. Likewise, studies have confirmed that MTX directly increases MDA levels in small intestinal tissue and causes oxidative stress by decreasing total GH levels.[28] In light of this information, studies have turned to antioxidants to reduce the side effects of MTX.[29,30] In the present study, MN effectively suppressed MTX-induced oxidation by

preventing the increase in MDA. This finding aligns with the results reported by Jahovic et al.[31], who administered a single intraperitoneal dose of 20 mg/kg MTX to rats. Their research revealed a decrease in glutathione levels in the blood, liver, kidney, and small intestine tissues and a significant increase in MPO and MDA levels. These increased levels of MDA and MPO serve as indicators of inflammatory response. Consistent with the literature, our study found that MPO levels were elevated in the group receiving MTX compared to the other groups. Our findings demonstrated that while MDA and MPO immunoreactivities increased after MTX administration, they decreased in all three intestinal segments after extract administration, suggesting that extract administration reduces MDA and MPO in both early and late periods. Our findings demonstrated a significant decrease in oxidative stress parameters following MN administration across all treatment groups and intestinal segments.

Evaluation of TAS reveals that oxidative reactions during hepatic metabolism of MTX lead to toxic side effects, causing hepatotoxicity and damage to rapidly proliferating normal tissues such as bone marrow and intestine. Measurements of oxidative stress, antioxidant defense, oxidant-antioxidant balance, and factors affecting this balance (TOS, TAS, and OSI) are available, and the levels of these factors can be measured and calculated separately.[32,33] In our study, statistical analysis of TOS and TAS levels measured to assess the antioxidant properties of MN revealed no significant results. This showed that MN had no TAS-enhancing effect. Despite the absence of significant changes in systemic TAS levels, the observed reduction in tissue oxidative stress markers suggests that the antioxidant effects of MN may be predominantly localized to intestinal tissue. Since mucositis primarily affects the gastrointestinal mucosa, MN may exert its protective effects locally without significantly altering systemic antioxidant capacity. Additionally, plasma TAS measurements may not be sufficiently sensitive to reflect regional oxidative changes in specific tissues. The duration of treatment and timing of sample collection may also have contributed to the lack of detectable systemic differences. Therefore, these findings suggest that MN exerts its protective effects mainly at the tissue level rather than through systemic antioxidant modulation.

Studies have shown that the mechanism of action of MN extract is related to its antioxidant effect, acting by disrupting the activation of the reactive form of MTX. This is due to an alkaloid, such as a flavonoid, contributing to the antioxidant effect of MN. Flavonoids had inhibitory effects on the death of normal cells induced by various oxidative stresses thanks to their strong antioxidant properties.[32] The findings of this study corroborated the literature and demonstrated that the most pronounced anti-inflammatory effect was observed in the MN group, in which inflammatory parameters declined.

To the best of our knowledge, no study has demonstrated a protective effect of MN against MTX-induced intestinal mucositis. In our study, we observed that MN was well tolerated in rats and produced the expected effect without causing abnormal changes in biochemical or immunohistochemical parameters. Morphometric and histopathologic findings from our study showed that MN exerted a protective effect against MTX-induced mucosal damage and promoted regeneration of the intestinal epithelium. Our findings are unique in that they provide the first evidence demonstrating the protective effect of MN against MTX-induced gastrointestinal toxicity at the tissue level. These results suggest that MN has the potential to protect against the adverse effects of MTX. However, further studies encompassing more extensive clinical applications are necessary to fully ascertain the efficacy and potential benefits of MN in this context.

Study Limitations

This study has several limitations. The MN extract was not phytochemically characterized [e.g., by high-pressure liquid chromatography (LC) or LC/tandem mass spectrometry], which may affect the reproducibility and the mechanistic interpretation. Only male rats were included, precluding the assessment of sex-related differences. The use of a single, relatively high dose limits the evaluation of dose-response relationships. Only two time points were assessed, which may not fully capture the dynamic course of mucositis and may increase the risk of type I error due to multiple comparisons. In addition, no formal power analysis was performed to determine sample size. Finally, functional outcome measures (e.g., intestinal permeability, nutrient absorption, and clinical indices of mucositis severity) were not evaluated, which may limit the physiological interpretation of the results.

CONCLUSION

The results of this study provide macroscopic, microscopic, and immunohistochemical evidence that MN's strong antioxidant and anti-inflammatory properties prevent MTX-induced intestinal damage. To the best of our knowledge, no studies demonstrate the protective effect of MN at the tissue level against MTX-induced intestinal mucositis. The morphometric and histopathologic findings in our study show that MN has a protective effect against MTX-induced mucosal damage and promotes regeneration of the intestinal epithelium. MN emerges as a promising agent in the prevention and treatment of MTX-induced oral and gastrointestinal mucositis.

Ethics

Ethics Committee Approval: The Manisa Celal Bayar University Local Ethics Committee for Animal Experiments granted ethical approval (approval number: 77.637.435-64, date: 08.11.2016).

Informed Consent: Since this is an animal experiment, consent is not required.

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Footnotes

Authorship Contributions

Surgical and Medical Practices: A.C.E., A.T.Y., S.V., İ.T., Concept: A.C.E., A.T.Y., H.G., Design: A.C.E., F.F., İ.T., H.G., Data Collection or Processing: A.C.E., A.T.Y., F.F., F.T., Analysis or Interpretation: F.F., S.V., İ.T., F.T., Literature Search: A.C.E., İ.T., H.G., Writing: A.C.E., A.T.Y., F.T.

Conflict of Interest: No conflict of interest was declared by the authors.

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