Aqueous parsley (*Petroselinum crispum*) extract ameliorated methotrexate-induced brain and small intestine damage in rats

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ABSTRACT

Methotrexate (MTX) is a widely used antiarthritic and chemotherapeutic agent known to cause damage to various tissues. This study investigated the potential protective effects of parsley extract against MTX-induced brain and intestinal tissue damage. Sprague-Dawley rats were divided into control, control + parsley, MTX, and MTX + parsley. MTX (20 mg/kg, i.p.) was administered to the MTX and MTX + parsley groups. The control + parsley, and MTX + parsley groups were administered 2 g/kg parsley extract by oral gavage for five consecutive days. After the fifth day, brain and small intestinal tissues were taken. Total protein, nitric oxide, lipid peroxidation, glutathione levels, tissue factor, superoxide dismutase, and glutathione S-transferase activities were determined in these tissues. The protein profiles of the tissues were evaluated using SDS polyacrylamide gel electrophoresis. Parsley administration caused a decrease in lipid peroxidation levels in both tissues of the MTX group. On the other hand, glutathione level, glutathione-S-transferase, and superoxide dismutase activities were found to be increased. On the other hand, parsley decreased the nitric oxide level which was increased in the intestinal tissues of the MTX group. There was no significant change in brain nitric oxide level and tissue factor activity between groups. MTX and parsley administration altered protein expression, leading to the appearance or disappearance of specific bands in intestinal and brain tissues. In conclusion, parsley alleviated MTX-induced damage in brain and intestinal tissues by reducing lipid peroxidation and modulating antioxidant defenses.

Introduction

Methotrexate is a cytotoxic agent widely used in the treatment of cancer and autoimmune diseases such as rheumatoid arthritis and psoriasis. The mechanism of action of methotrexate (MTX) is based on the inhibition of dihydrofolate reductase and other related enzymes involved in purine and thymidine synthesis and ultimately inhibition of DNA, RNA, and protein synthesis. The cessation of these biosynthesis prevents biochemical processes such as vital ATP molecule synthesis and cell division (2, 3). However, as with other chemotherapeutic agents, its side effects affect the quality of life of patients and cause limitations in the use of the drug.

Studies have shown that MTX treatment can cause cognitive impairments such as decreased memory and learning by causing neuroinflammation and oxidative stress in rats (36, 40, 46). MTX-induced intestinal irritation sends signals to the brain via the gut-brain axis, activating microglia and increasing cytokines, which eventually leads to neuroinflammation (19, 37).

It has been reported that the cause of MTX-induced damage in the brain and intestine, as in many tissues, is due to oxidative stress. Although the mechanism of neurotoxicity is not clear, it is stated that the apoptosis pathway stimulated by the disruption of folate metabolism caused by MTX, disorders in myelin synthesis, and oxidative stress may be involved (30). It has been shown that reactive oxygen species have some roles in the formation of this damage. MTX causes a decrease in nicotinamide adenine dinucleotide phosphate (NADPH) within the cell. Since NADPH is a cofactor of glutathione reductase, which is involved in glutathione (GSH) homeostasis, this causes a decrease in GSH levels (18, 21). In addition, the reactive species formed disrupt the structures of molecules such as proteins, lipids, and nucleic acids, causing DNA damage and lipid peroxidation. Since the cells cannot adequately defend against this oxidative damage, MTX-induced tissue damage occurs (21). Similarly, studies have shown that MTX-induced oxidative stress also plays a role in intestinal toxicity (29, 32, 35). One of the critical pathways implicated in MTX-induced toxicity is the activation of tissue factor (TF), a transmembrane glycoprotein and a key initiator of the extrinsic coagulation cascade. Although TF is primarily known for its role in thrombosis, emerging evidence suggests that it also plays a significant role in inflammation, cell injury, and tissue damage. Elevated TF expression has been associated with the pathophysiology of several diseases, including cancer, cardiovascular disorders, and organ-specific damage (8). Studies indicate that TF activation leads to inflammatory responses, endothelial dysfunction, and increased vascular permeability, contributing to tissue injury (9).

Parsley (*Petroselinum crispum*), a green plant that is a member of the Umbelliferae family, has antioxidant, antidiabetic, anti-inflammatory, and antiapoptotic properties (11). Phytochemical analyses have shown that it contains many molecules with biological properties, such as flavonoids, coumarins, carotenoids, apiol, myristicin, phthalides, sesquiterpenes, monoterpenes and ascorbic acid (44). Among these molecules, flavonoids, carotenoids, tocopherols, essential oils, and ascorbic acid, preventing free radical formation, exhibit antioxidant properties, while the essential oils in their content play a role in suppressing autoimmune and chronic inflammatory disorders and allergies (24). Just as there is a close relationship between changes in the intestinal microbiota and various neurological disorders, there is also a close relationship between changes in the intestinal microbiota and chemobrain (22, 47). MTX administration in rats alters the link between the small intestine and the brain, principally by inducing intestinal inflammation, disrupting gut microbiota, and resulting in neuroinflammation and cognitive impairment (5, 37). Understanding these mechanisms indicates the role of the gut-brain axis in mediating MTX side effects and proposes possible treatment methods to maintain gut and brain health. Therefore, this study aims to evaluate the effect of aqueous parsley extract on the rat intestinal and brain oxidant-antioxidant balance disrupted by MTX administration. The selected parameters, including the specific biochemical markers assessed—such as total protein, nitric oxide, lipid peroxidation, glutathione levels, and the activities of tissue factor, superoxide dismutase, and glutathione S-transferase—were used to evaluate the extent of tissue injury and the protective effects of parsley. By analyzing these parameters, the study provides valuable insights into the mechanisms through which parsley mitigates MTX-induced oxidative stress and tissue injury.

Materials and Methods

Preparation of Aqueous Parsley Extract: The aqueous parsley extract was prepared at the Istanbul University Cerrahpaşa Faculty of Engineering, Department of Biochemistry.

The leaves of the parsley plant purchased from a local greengrocer were thoroughly washed and dried at room temperature for 3 days and stored in cellophane bags. Dried parsley leaves (100 g) were extracted with 1000 ml of distilled water and boiled for 30 minutes. The extract was filtered, and the solvent in the obtained extract was evaporated to dryness under reduced pressure in a rotary evaporator (12). The obtained powdered parsley extract was stored at -20 °C.

Experimental Animal Model: This experimental study was conducted with the permission of the Marmara University Animal Experiments Local Ethics Committee dated 11.01.2022 and protocol numbered 02.2022mar A number of thirty-two female and male Sprague Dawley rats of three months old weighing 200-300 g were used in the study. The rats were kept in conventional cages with a condition of a 12-hour light-dark cycle and a room temperature of 22 ± 2 °C. The rats were fed standard pellet feed and drinking water ad libitum throughout the experimental period.

Rats were divided into control, control+parsley, methotrexate (MTX), and MTX+parsley groups. Each group had 8 rats. In the control group, to imitate methotrexate administration, saline was given to rats intraperitoneally. To imitate the oral gavage method, 0.5 ml of drinking water was given by oral gavage for 5 days. In the control+parsley group, rats were given 2 g/kg parsley extract dissolved in 0.5 ml of water by the oral gavage method for 5 days after a single dose of intraperitoneal saline administration. MTX was dissolved in physiological saline and administered to the animals as

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a single dose of 20 mg/kg (intraperitoneally) in the MTX group (4). 0.5 ml of drinking water was also given by oral gavage for 5 days. In the MTX+parsley group, a single dose of 20 mg/kg MTX was administered intraperitoneally, and 2 g/kg parsley extract was also administered to the rats by oral gavage for 5 days.

Rats in all groups were decapitated, and their brain and small intestine tissues were taken at the end of the fifth day. 10% tissue homogenates were prepared using physiologic saline solution for biochemical analysis.

Determination of Lipid Peroxidation: Malondialdehyde (MDA) is one of the end products formed through the decomposition of lipid peroxidation products. MDA level was measured by using the method of Ledwozvy et al. (23). In this method, the sample undergoes a reaction with TBA under acidic conditions, resulting in the formation of an MDA-TBA complex. The intensity of the pink color formed is proportional to the MDA concentration and can be quantified by measuring absorbance at around 532 nm. Brain and small intestine MDA levels were expressed as nmol MDA/g tissue.

Determination of Glutathione Level, Superoxide Dismutase, and Glutathione-S-Transferase Activity: Brain and small intestine glutathione (GSH) levels were determined by the modified Beutler's method (6). In this method, after the addition of a precipitation solution to the homogenate, the sample is centrifuged. The supernatant was treated with Na_2HPO_4 and Ellman's reagent. Absorbance was measured at 412 nm. GSH levels were expressed as mg GSH/g tissue.

Brain and small intestine superoxide dismutase (SOD) activities were determined by the method of Mylroie et al. (28). The method involves measuring the absorbance of potassium phosphate buffer, riboflavin, o-dianisidine, and tissue extract. The mixture was illuminated with fluorescent light and absorbance was measured at 460 nm. SOD activities were expressed as U/g tissue.

Determination of GST activity is based on the principle of measuring the absorbance at 340 nm of the product formed by the conjugation of glutathione (GSH) and 1-chloro-2,4-dinitro-benzene (CDNB) by spectrophotometric method (16).

Determination of Total Protein, Nitric Oxide Levels, and Tissue Factor Activity: Brain and small intestine total protein levels were determined by the method of Lowry et al. (45). This method involves the reaction of protein with a copper ion in an alkaline solution, followed by the addition of a Folin-Ciocalteu reagent. The resulting color change is proportional to the protein concentration. The total protein levels were expressed as mg protein/g tissue.

The method of Miranda et al (27). was used for the detection of brain and small intestine NO levels. The method involves measuring the conversion of NO to its stable metabolite, nitrite, which can be quantified. The process typically includes the addition of reagents that react with nitrite, producing a color change, and the absorbance is measured at 540 nm. NO levels were expressed as µmol NO/g tissue.

Brain Tissue factor (TF) activity was determined by using the modified Quick's one-stage method (20). This method involves measuring the time it takes for blood clotting to occur in the presence of a tissue extract. This method assesses the ability of TF to initiate the coagulation cascade by adding a plasma sample to the tissue extract and measuring the clotting time. The clotting time is inversely related to TF activity, with shorter clotting times indicating higher TF activity.

Brain and Small Intestine Protein Electrophoresis: An electrophoretic examination of brain and small intestine proteins was carried out by using Laemmli SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (17). SDS–PAGE was performed by using the BIO-RAD Mini Protean Precast II Dual Slab Gel Apparatus (BIO-RAD, USA). Mini PAGE gels (any kD precast polyacrylamide gel, 8.6 × 6.7 cm [W × L], Catalog Number: 4569033, BIO-RAD, USA) were used for protein electrophoresis. High-resolution photographs of the gels were taken using a Canon EOS 700D camera with an 18–55 lens to evaluate protein bands after electrophoresis, and the images were exported as JPEG files. Densitometric graphs of protein bands were plotted using ImageJ software (33).

Statistical Analysis: GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) was used for the statistical analysis of the data. The normality of the data was checked using the Shapiro-Wilk test before applying parametric tests. All results were presented as mean and standard error of mean (SEM). One-way analysis of variance (ANOVA) (post-hoc Tukey test) was used for comparison between groups. $P<0.05$ was considered statistically significant. Statistical power analysis was performed on the small intestine and brain NO levels using Faul et al.'s (13) method.

Results

Small Intestine Results: Significant decreases were found in the total protein and glutathione levels, glutathione-S-transferase, and superoxide dismutase activities of the methotrexate group compared to the C and C+Parsley groups. Parsley administration to the MTX group significantly increased these intestinal parameters (Figure 1). Malondialdehyde and nitric oxide levels significantly increased in the MTX group compared to the C and

C+Parsley groups. Parsley administration to the MTX group significantly decreased malondialdehyde and nitric oxide levels. Parsley administration to the control group also significantly decreased malondialdehyde and nitric oxide levels and significantly increased glutathione levels, glutathione-S-transferase, and superoxide dismutase activities and did not change total protein levels. The power analysis for the small intestine NO levels was conducted with a specific effect size, sample size (n = 32 per group), and a significance level of $\alpha = 0.05$. The resulting power value of 0.95 indicates that there is a 95% probability of correctly rejecting the null hypothesis, assuming a true effect exists. This high power ensures that the study was well-equipped to detect significant differences in NO levels.

Brain Results: Significant decreases were found in the brain glutathione level, glutathione-S-transferase, and superoxide dismutase activities of the methotrexate group compared to the C and C+Parsley groups. Parsley administration to the MTX group significantly increased these parameters in brain tissue (Figure 2). Brain malondialdehyde levels significantly increased in the MTX group compared to the C and C+Parsley groups. Parsley administration to the MTX group did not change the MDA level. No significant differences were detected between all the groups in nitric oxide levels and tissue factor activities of the brain tissue. Parsley administration to the control group significantly increased glutathione level and superoxide dismutase activity and did not change the other parameters. The power analysis for the brain NO levels was conducted with a specific effect size, sample size (n = 32 per group), and a significance level of $\alpha = 0.05$. The resulting power value of 0.95 indicates that there is a 95% probability of correctly rejecting the null hypothesis, assuming a true effect exists.

Electrophoretic Evaluation of Small Intestine and Brain: According to the electrophoresis results of intestinal tissue, protein bands with molecular weights of approximately 80, 90, and 100 kDa were not found in the C and C+Parsley groups but were seen in the MTX and MTX+Parsley groups. It was determined that the 66 kDa protein decreased with the administration of MTX and that the parsley administration to the MTX group caused this band to decrease even more. The 36 and 45 kDa proteins were present in the C, C+Parsley, and MTX groups but disappeared in the MTX+Parsley group (Figure 3).

According to the electrophoretic evaluation of brain tissue, proteins weighing 24, 36, and 45 kDa disappeared in the parsley-administered MTX group, while they remained unchanged in the other groups (Figure 4).

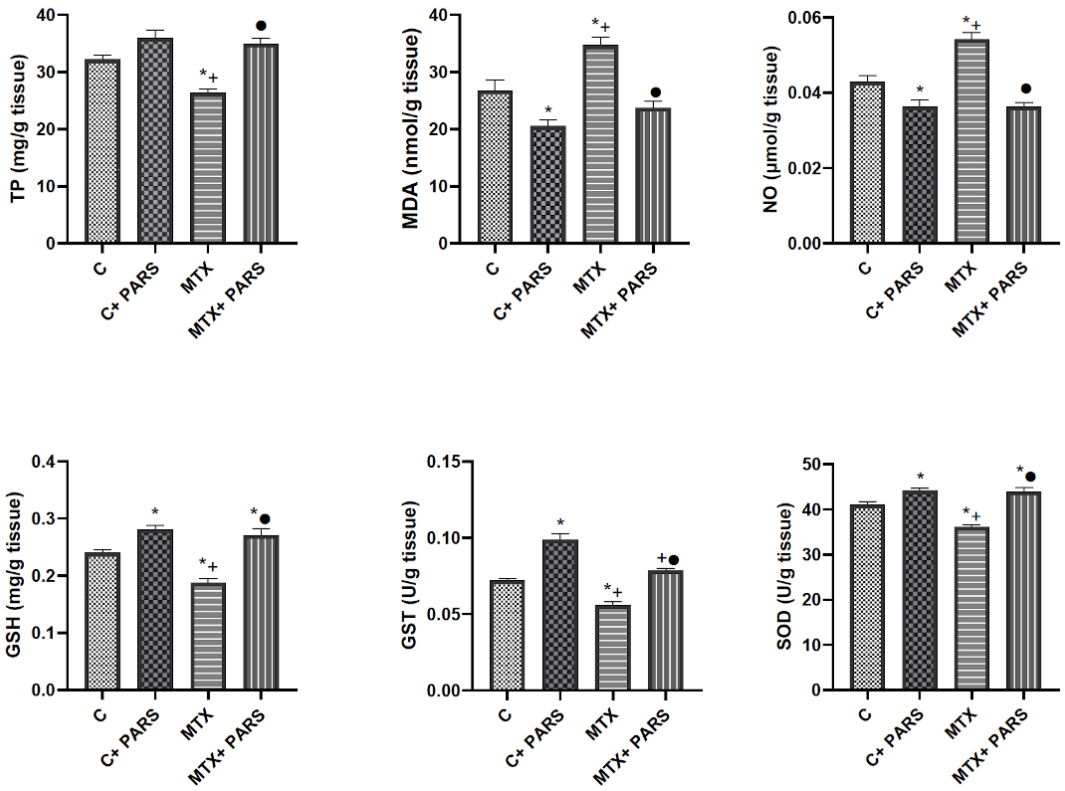

Figure 1. Biochemical results of small intestine tissue

C: Control, **C+Parsley**: Control+parsley, **MTX**: Methotrexate, **MTX+Parsley**: Methotrexate+ parsley group, results were presented as Mean ± Standard Deviation, n=8 in each group. (*) P<0.05 is significant according to the C group, (+) P<0.05 is significant according to C+ Parsley, (●) P<0.05 is significant according to the MTX group.

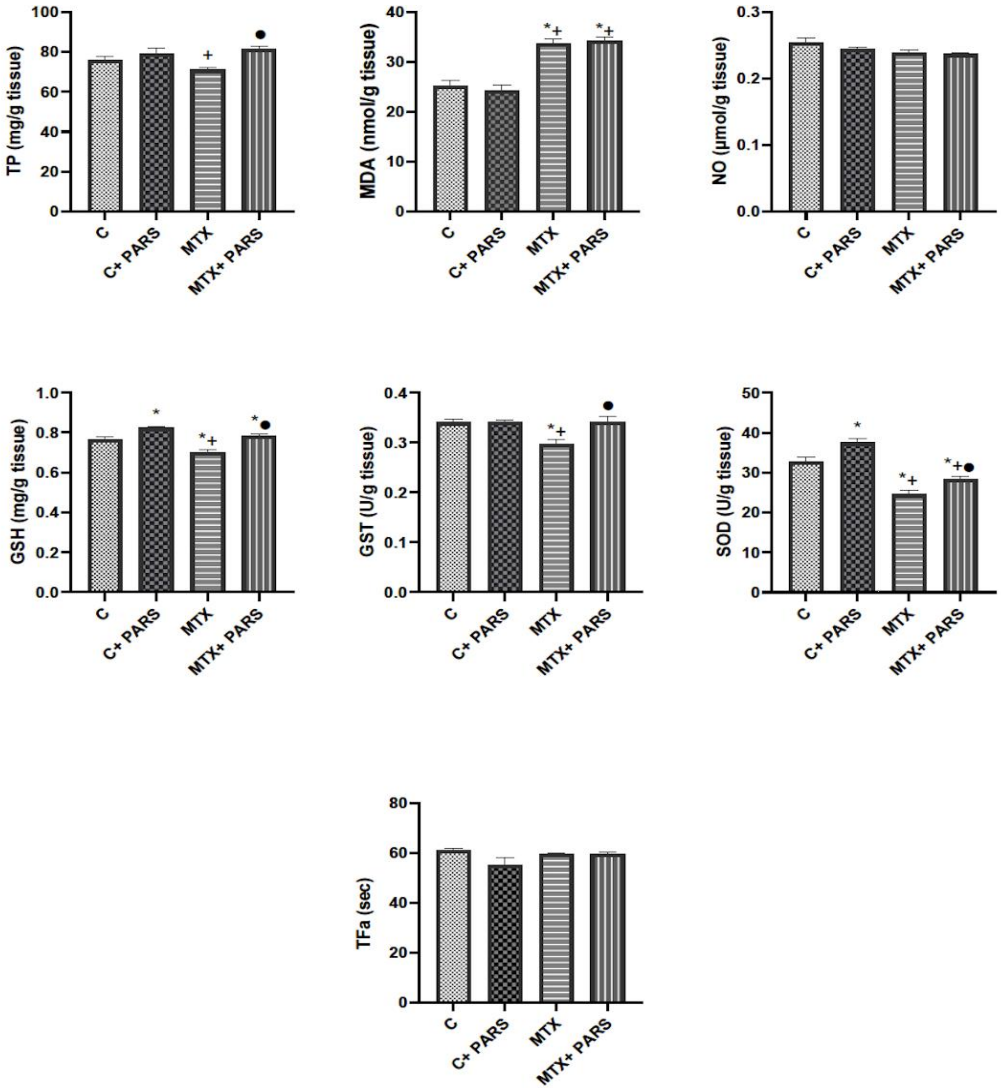

Figure 2. Biochemical results of brain tissue.

C: Control, **C+Parsley**: Control+parsley, **MTX**: Methotrexate, **MTX+Parsley**: Methotrexate+ parsley group, results were presented as Mean ± Standard Deviation, n=8 in each group. (*) P<0.05 is significant according to the C group, (+) P<0.05 is significant according to C+ Parsley, (●) P<0.05 is significant according to the MTX group.

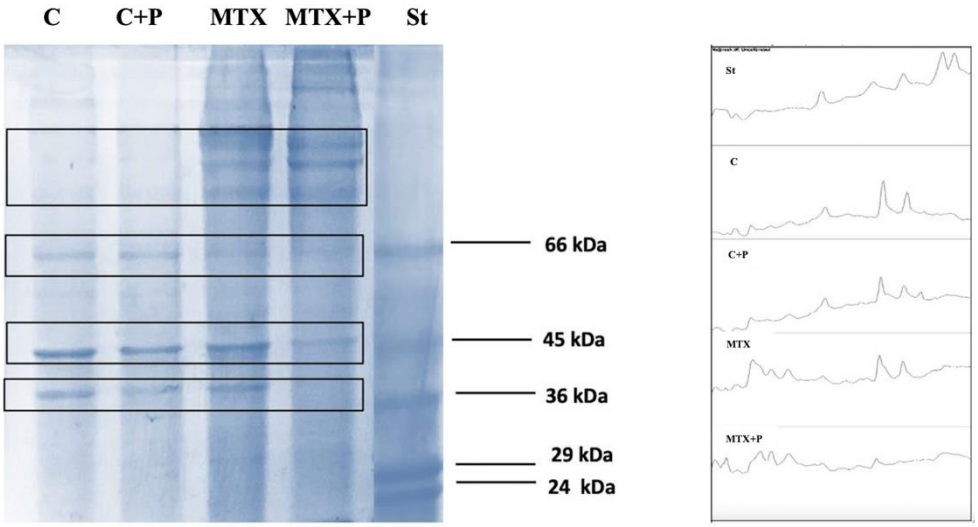

Figure 3. Electrophoretic evaluation of small intestine tissue proteins**.**

C: Control, **C+P**: Control+Parsley, **MTX**: Methotrexate, **MTX+P**: Methotrexate+Parsley group, **St:** Standard protein marker.

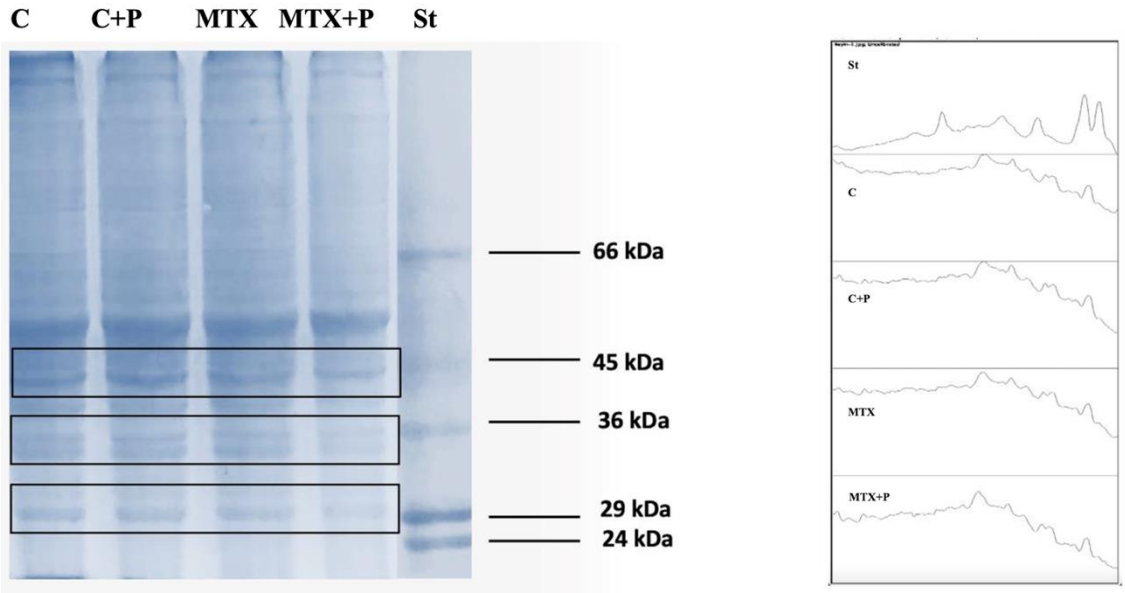

Figure 4. Electrophoretic evaluation of brain tissue proteins.
C: Control, **C+P**: Control+Parsley, **MTX**: Methotrexate, **MTX+P**: Methotrexate+Parsley group, **St:** Standard protein marker.

Discussion and Conclusion

This study provides new insights into the biochemical and physiological effects of methotrexate (MTX) administration on brain and small intestine tissues. A significant increase was observed in MDA levels, accompanied by a decrease in glutathione levels, superoxide dismutase activity, and glutathione-S-transferase activity, indicating oxidative stress and impaired antioxidant defense mechanisms. Yılmaz et al. (48) have reported that a single dose of intraperitoneal MTX (20 mg/kg) administration induced brain damage by increasing MDA and decreasing antioxidant parameters (GSH, GST, and SOD). They also found that MTX administration caused an increase in the NO levels of intestinal tissue but did not cause any significant change in the brain. Rtibi et al. (32) have found an increase in MDA levels and a decrease in antioxidant parameters (SOD, CAT, and GPx) of intestinal tissue of rats administered orally 100 mg/kg MTX. Similarly, El-Baghdadi et al. (10)observed increased NO and MDA levels and decreased GSH levels in the small intestine tissue. It was also revealed that MTX administration can increase the mRNA expression and synthesis of NOS enzymes (34).

In this study, in line with the literature, MTX administration increased intestinal and brain MDA levels and decreased GSH levels and SOD and GST activities. In addition, MTX administration increased NO levels in the small intestine but did not change NO levels in the brain. Tissue factor activity is another parameter examined in this study. Tissue factor is a coagulation protein involved in the extrinsic pathway of the coagulation mechanism (14). In this study, TF activity could not be detected in the intestinal tissue. In brain tissue, MTX administration did not change TF activity in all groups.

Many studies have examined the effects of various antioxidant supplements and substances, such as whey proteins, naringenin, bromelain, L-carnitine, melatonin, apricots, and beta-carotene, on MTX-induced oxidative stress-induced damage (15, 25, 38, 39, 41, 42). In this study, the effects of parsley, which has antioxidant properties thanks to molecules such as apiol, myristicin, apiin, luteolin, and beta-carotene in its content, against intestinal and brain damage caused by experimental MTX were investigated.

Parsley has antidiabetic, antibacterial, antioxidant, anticoagulant, and immune system-strengthening properties due to many phytochemicals in its structure (24, 31). Ertaş et al. (11) stated that parsley prevents lipid peroxidation in liver damage caused by MTX by increasing GSH levels. In the study conducted by Maodaa et al. (26), it was determined that parsley reduces lipid peroxidation and increases GSH levels and glutathione peroxidase enzyme activity in oxidative damage caused by cadmium in the brain. In another study conducted on the brain, it was stated that parsley extract reduced MDA levels and increased the activities of antioxidant enzymes SOD and glutathione peroxidase in brain damage caused by D-galactose (43). It has also been found that parsley has a gastroprotective effect in a gastric ulcer model created in intestinal tissue by pyloric ligation (1). In this study, when parsley extract was given to rats treated with MTX, a decrease in MDA levels was detected in the small intestine and brain tissues. In addition, parsley extract

administration to the MTX group caused a significant decrease in intestinal NO levels but did not change brain NO levels and TF activity.

Boukhettala et al (7) . stated that MTX treatment altered the intestinal mucosa and protein metabolism by decreasing protein synthesis and increasing proteolysis mediated by the lysosomal pathway. Methotrexate can cause villous atrophy. Therefore, morphological changes such as decreased villus length and increased crypt depth can be observed. In this study, when the protein profile of intestinal and brain tissues was examined with SDS-PAGE, a decrease in some protein bands and an increase in some protein bands were detected in both tissues in the MTX and parsley-treated MTX groups. In the small intestine tissue, protein bands with molecular weights of approximately 80, 90, and 100 kDa were not found in the C and C+Parsley groups but were seen in the MTX and MTX+Parsley groups. It was also determined that the 60-65 kDa protein band decreased with the administration of MTX and that the parsley administration to the MTX group caused this band to decrease even more. Two protein bands (36 kDa and 45 kDa) of the C, C+Parsley, and MTX groups also disappeared in the MTX+Parsley group (Figure 1). The exact causes and consequences of changes in proteins can be examined in detail and determine the context of changes in their expressions. According to the electrophoretic evaluation of brain tissue, proteins weighing 24, 36, and 45 kDa disappeared in the parsley-administered MTX group, while they remained unchanged in the other groups (Figure 2).

In conclusion, it was determined that parsley has a protective effect against MTX-induced oxidative damage in small intestine and brain tissues. It has the potential to improve gut-brain axis communication altered by MTX administration. Further studies are needed to investigate whether the effects of parsley are dose-dependent, as this could provide valuable insights into its therapeutic potential and provide more clarity on how it interacts with methotrexate.

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Ethical Statement

This study was carried out after the animal experiment was approved by Marmara University, Animal Experiments Local Ethics Committee (Decision Number: 02.2022mar)

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

ED was responsible for organizing and conducting the biochemical experiments. SYK, GGS, and ET performed biochemical analyses. ÖS, RY, and GŞ conceived and designed the experiments. TTA supervised the study, contributed to manuscript writing, and interpreted the results. All authors provided critical feedback and contributed to shaping the research, analysis, and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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