DOI: 10.24938/kutfd.1347778 Kırıkkale Üniversitesi Tıp Fakültesi Dergisi 2023;25(3):455-462
Original Article *Özgün Araştırma*

THE AMELIORATING EFFECTS OF BILBERRY ON METHOTREXATE-INDUCED INTESTINAL INJURY

Metotreksatın İndüklediği İntestinal Hasar Üzerine Yaban Mersininin İyileştirici Etkileri

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ABSTRACT

Objective: Methotrexate (MTX) is a folic acid analogue chemotherapeutic used in the treatment of some malignant tumors and autoimmune diseases. In addition to its antitumoral properties, it can also exhibit toxic effects on the kidney and intestines. Bilberry (BB) fruit is a potent natural antioxidant rich in anthocyanidins. The purpose of this study was to investigate the potential ameliorating effects of BB against MTX-induced intestinal damage using biochemical and histological methods.

Material and Methods: Twenty-one adult female Sprague Dawley rats were divided into three equal groups. No procedure was performed on the control group (ControlG), while the MTX group (MTXG) and MTX+BB extract group (MTX+BBG) received a single intraperitoneal dose of 30 mg/kg MTX on the first day of the experiment. MTX+BBG also received 200 mg/kg BB extract by oral gavage once daily for five days starting on the first day of the experiment. Half the intestinal tissues removed from the duodenal region at the experiment were used for biochemical evaluation, and the other half for histological examination.

Results: Malondialdehyde (MDA), total oxidant status (TOS), oxidative stress index (OSI), and 8-oxo-2′-deoxyguanosine (8-OHdG) values were all higher in MTXG intestinal tissues than in ControlG. MDA, TOS, OSI, and 8-OHdG values were lower in MTX+BBG than in MTXG. In addition, caspase-3 levels in MTXG were higher than those in both ControlG and MTX+BBG, while total antioxidant status (TAS) values were lower. In terms of histology, villous hemorrhage, inflammatory cell infiltration, fusion, and degeneration in the villus epithelium were present in MTXG intestinal tissue, and the total damage score was also high. Improvement in all these parameters was present in MTX+BBG.

Conclusion: Orally administered BB extract can improve MTX-induced intestinal damage through its antioxidant and anti-inflammatory effects.

Keywords: *Antioxidant, intestine, methotrexate, oxidative stress, bilberry.*

ÖZ

Amaç: Metotreksat (MTX), bazı malign tümörlerin ve otoimmün hastalıkların tedavisinde kullanılan folik asit analoğu bir kemoterapötiktir. Antitümoral etkilerinin yanında karaciğer, böbrek ve bağırsaklar üzerine toksik etkiler de gösterebilir. Yaban mersini (YM), güçlü ve doğal bir antioksidan olan antosiyaninlerden zengin bir meyvedir. Bu çalışma; MTX ile indüklenen intestinal hasara karşı YM ekstraktının olası iyileştirici etkilerini biyokimyasal ve histolojik olarak araştırmayı amaçlamaktadır.

Gereç ve Yöntemler: Çalışmada kullanılan 21 adet Spraque Dawley ırkı erişkin dişi sıçan, 3 eşit gruba ayrıldı. Kontrol grubu (KontrolG)'na hiçbir işlem uygulanmadı. MTX grubu (MTXG)'na ve MTX+YM ekstraktı grubu (MTX+YMG)'na deneyin ilk günü tek doz 30 mg/kg MTX intraperitoneal olarak uygulandı. MTX+YMG'na, ilave olarak deneyin ilk gününden itibaren 5 gün, günde bir kez oral gavaj ile 200 mg/kg YM ektraktı uygulandı. Deney sonunda duodenal bölgeden alınan intestinal dokuların yarısı biyokimyasal, diğer yarısı histolojik olarak değerlendirildi.

Bulgular: MTXG bağırsak dokularında; malondialdehid (MDA), total oksidan kapasite (TOS), oksidatif stres indeksi (OSI) ve 8-oxo-2′-deoxyguanosine (8-OHdG) değerleri KontrolG'na göre yüksekti. MTX+YMG'nun MDA, TOS, OSI ve 8-OHdG değerleri ise MTXG'na göre azalmıştı. İlave olarak MTXG'nun Kaspaz 3 seviyesi hem ControlG'na hem MTX+YMG'na göre yüksek; total antioksidan durum (TAS) seviyeleri ise düşüktü. Histolojik olarak MTXG bağırsak dokusunun villuslarında hemoraji, inflamatuvar hücre infiltrasyonu, füzyon ve villus epitelinde dejenerasyon vardı ve total hasar skoru da yüksekti. MTX+YMG'nda ise tüm bu patolojilerde hafifleme mevcuttu.

Sonuç: Oral olarak uygulanan YM ekstraktı; antioksidan ve antienflamatuvar etkileri ile MTX'in indüklediği bağırsak hasarını iyileştirebilir.

Anahtar Kelimeler: *Antioksidan, bağırsak, metotreksat, oksidatif stress, yaban mersini.*

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Received / Geliş Tarihi: 23.08.2023 **Accepted** / Kabul Tarihi: 21.09.2023

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Doi: 10.24938/kutfd.1347778

INTRODUCTION

Gastrointestinal toxicity is one of the toxic effects, which still cannot be fully prevented, of several chemotherapeutics, including methotrexate (MTX) (1). MTX is an antimetabolite chemotherapeutic used in the treatment of autoimmune diseases such as rheumatoid arthritis, vasculitis, and psoriasis, in addition to several malignant diseases (2,3). A structural analogue of folic acid, MTX blocks the enzyme dihydrofolate reductase and leads to the death of cancer cells by impairing DNA and RNA synthesis in the cell (3,4). However, similarly to other chemotherapeutics used in the treatment of cancer, MTX can damage healthy cells in the organism as well as cancerous cells (1). In addition to its therapeutic effects, MTX can also therefore exhibit undesirable toxic effects in several tissues, including the liver, kidneys, and intestines (5-8). Several studies have shown that MTX causes pathologies such as increased oxidative stress in the intestines, apoptosis, epithelial damage, inflammation, and mucositis (7,9-12). Although the mechanism involved in these toxic effects is not fully understood, it has been attributed to MTX impairing the synthesis of DNA and RNA in intestinal cells, its increasing reactive oxygen species (ROS) that give rise to oxidative stress, and thus resulting in a decrease in cell numbers and disruption in the mucosal epithelium (2).

Bilberry (BB, Vaccinium myrtillus) otherwise known as whortleberry, is a blue-back fruit, and a source of natural antioxidants that can be consumed in various forms: fresh, dry, tinned, or as alcoholic or non-alcoholic beverages (13,14). It contains numerous antioxidant compounds, including quercetin, resveratrol, catechins, vitamin C, phenolic acid, and anthocyanins (13,15). Due to these compounds, and particularly anthocyanins, BB exhibits antioxidant and anti-inflammatory effects (13,16,17).

To the best of our knowledge, no previous studies have investigated the effects of BB against MTX-induced intestinal damage. The purpose of this research was therefore to investigate the protective effects of BB, with its antioxidant and anti-inflammatory properties, against intestinal damage induced with MTX in rats.

MATERIALS AND METHODS

Animal rights were respected in accordance with the principles of the Guide for the Care and Use of Laboratory Animals. Approval for the study was granted by the Karadeniz Technical University Animal Experiments Local Ethics Committee, Türkiye, under number 2022/50. Twenty-one adult female Sprague Dawley rats were randomly assigned to three groups of seven animals each. Throughout the experiment, all rats were housed in the same room in a laboratory at a mean temperature of 22±2°C, 50%±5 relative humidity, and a 12-hour dark:light cycle. No procedure was performed on the control group (ControlG), while the MTX group (MTXG) and MTX+BB extract group (MTX+BBG) received a single intraperitoneal dose of 30 mg/kg MTX on the first day of the experiment (7). MTX+BBG also received 200 mg/kg BB extract once daily for five days starting on the first day of the experiment by oral gavage.

BB Extract preparation

Bilberry fruits, collected from a rural area of Trabzon province in Türkiye, were first dried for 20 days at 45˚C and then powdered using a laboratory mill (Retsch ZM200, Haan, Germany). Next, 3 g of powder was mixed with 30 mL pure ethanol and vortexed. The ethanolic mixture was then incubated for 24 hours at 45˚C with continuous shaking at 150 rpm, and was then centrifuged at 1800xg for 10 min. The resulting supernatant was first passed through filter paper, then passed through a 0.2-µm filter, and stored in the dark at 4˚C for use in the experiment (18).

Tissue collection

On the sixth day of the experiment, all rats received 10 mg/kg xylazine hydrochloride (Rompun: Bayer, Leverkusen, Germany) and 50 mg/kg ketamine hydrochloride (Ketalar: Pfizer, İstanbul, Türkiye) via the intraperitoneal route for deep anesthesia. A midline abdominal incision was then made, through which the duodenal region of the small intestine was excised, after

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which all rats were sacrificed by exsanguination. One part of the intestinal tissue was washed with saline solution and stored in micro-volume tubes at -80°C for biochemical evaluation, while the other part was stored in 10% formalin in glass jars for histopathological examination.

Histological evaluations

Tissues kept in 10% formalin solution for fixation were subsequently dehydrated by being passed through increasing alcohol series. They were then rendered transparent with xylene and embedded in paraffin. Serial sections 5 µm in thickness were taken from the paraffin blocks using a microtome (Leica RM 2255, Leica Instruments, Nussloch, Germany). These were stained with hematoxylin-eosin (HE) and Masson's trichrome and covered. These stained sections were next subjected to histopathological examination under a light microscope (Olympus BX 51, Japan) and photographed using a digital camera (Olympus, DP 71, Japan) integrated with the microscope. Five randomly selected areas on each section were scored semiquantitatively from 0 to 3 in terms of each of the following pathological changes; superficial epithelial degeneration, villus fusion, hemorrhage, and inflammatory cell infiltration (in other words, inflammation) (0: none, 1: mild, 2: moderate, and 3: severe) (7). Increases in connective tissue and collagen were also evaluated qualitatively in sections stained with Masson's trichrome.

Biochemical analysis

Biochemical parameters were measured from supernatants obtained following the homogenization in phosphate buffer and centrifugation of the duodenal tissues. Protein levels in tissue samples were determined using an appropriate protein assay kit (Thermo Scientific Pierce BCA Protein Assay Kit, catalogue no. 23227, Rockford, lL, USA) in line with the manufacturer's instructions. The levels of biochemical parameters in tissues measured using ELISA kits were expressed as mg/protein by proportioning the calculated sample protein quantities. Tissue malondialdehyde (MDA) and 8-oxo-2′-deoxyguanosine (8-OHdG) levels were determined using ELISA kits (YLBiont, catalogue no. YLA0029RA and catalogue no. YLA0061RA, respectively, Shanghai, PRC) as recommended by the manufacturer. The results were expressed as nmol/mg protein and ng/mg protein, respectively. Total oxidant status (TOS) and total antioxidant status (TAS) in tissue specimens were determined using colorimetric kits (Rel Assay Diagnostics, catalogue no. RL0024 and RL0017, respectively, Gaziantep, Türkiye). The results were expressed as µmol H_2O_2 equivalent/L and mmol TE/L units. OSI values were calculated using the formula OSI=[(TOS, µmol H_2O_2 equivalent/L)/(TAS, µmol TE/L)]x100 (19). Tissue specimens' superoxide dismutase (SOD) and caspase-3 levels were measured using ELISA kits (YLBiont, catalogue no. YLA0115RA and YLA0017RA, respectively, Shanghai, PRC). The results were expressed as ng/mg protein.

Statistical analysis

Statistical analyses were performed on SPSS version 22.0 software. Parametric data were analyzed using ANOVA and the post-hoc Tukey test, and non-parametric data using the Kruskal-Wallis test. The results were expressed as mean±standard deviation (SD), median (minimum-maximum) and p values $p<0.05$ were regarded as statistically significant.

RESULTS

Biochemical analysis results

The ANOVA test revealed significant differences between the groups in all biochemical parameters ($p<0.05$) apart from SOD values ($p=0.93$). The post-hoc Tukey test used for pairwise group comparisons showed that MDA, TOS, OSI, and 8-OHdG values were significantly higher in MTXG than in ControlG ($p<0.001$, $p=0.003$, $p<0.001$, and $p=0.01$, respectively). Similarly, MTXG caspase-3 levels were higher than those in ControlG and MTX+BBG ($p=0.04$ and $p=0.014$, respectively). MDA, TOS, OSI, and 8-OHdG values decreased in MTX+BBG compared to MTXG ($p=0.005$, $p=0.02$, $p=0.001$, and $p=0.01$, respectively). MTXG TAS values were significantly lower than those

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of both ControlG and MTX+BBG (p<0.001 and p=0.03, respectively) (Table1).

Table 1: Biochemical analysis results of tissues

	ControlG	MTXG	MTX+BBG	p (ANOVA)
MDA (nmol/mg protein)*	4.09±1.40	7.95±1.26**	5.02±1.76***	<0.001
8-OHdG (ng/mg protein)*	0.12±0.04	0.20±0.04**	0.11±0.02***	0.008
SOD (ng/mg protein)*	0.52±0.24	0.48±0.18	0.49±0.10	0.93
TAS (mmol TE/L)*	1.75±0.35	1.02±0.20**	1.42±0.23***	<0.001
TOS (µmol H_2O_2 equivalent/L)*	8.82±3.27	17.9±4.39**	11.03±1.92***	0.003
OSI*	0.51±0.23	1.82±0.58**	0.81±0.42***	<0.001
Caspase-3 (ng/mg protein)*	0.10±0.05	0.18±0.06**	0.09±0.03***	0.011

MDA: Malondialdehyde, 8-OHdG: 8-oxo-2′-deoxyguanosine, SOD: Superoxide dismutase, TAS: Total antioxidant status, TOS: Total oxidant status, OSI: Oxidative stress index. ControlG: Control group, MTXG: Methotrexate group MTX+BBG: Methotrexate+bilberry extract group, Data expressed as mean±SD and p values p<0.05 were regarded as statiscally significant.

* mean ± standart deviation

** p<0.05 compared with ControlG according to post-hoc Tukey test.

*** p<0.05 compared with MTXG according to post-hoc Tukey test.

Histopathological results

Villi, covered with striated border single layer of prismatic epithelium and goblet cells between them, extended into the lumen in the form of finger-like projections were present in ControlG. Brunner's glands were visible in the tunica submucosa (Figure 1A, and D).

In MTXG, however, the villus structure was impaired, with shortening and blunting of the villus tips. The Kruskal Wallis test revealed significant differences between the groups in all histological parameters (p<0.05). The post-hoc Dunn's test used for pairwise group comparisons revealed the increase of villous hemorrhage, inflammatory cell infiltration, fusion, and degeneration in the villus epithelium in MTXG compared to ControlG, and the total damage score was higher (p=0.001, p<0.01, p=0.002, p=0.004, and p=0.001, respectively) (Figure 1B and E). In MTX+BBG, all these pathological findings and total damage scores decreased compared to MTXG, although statistical significance was only present in the hemorrhage parameter (p=0.02) (Figure 1C and F) (Table 2). No significant difference was observed between the groups in terms of collagen and connective tissue density in Masson trichrome stained sections (Figure 1A, B and C).

Table 2: Histopathological damage score results of tissues

	Control	MTXG	MTX+BBG	p (Kruskal Wallis)
Epithelial degeneration*	1 (0-2)	2 (2-3)**	2 (1-2)	0.002
Villous fusion*	0 (0-1)	2 (2-3)**	1 (0-2)	<0.001
Hemorrhage*	0 (0-1)	1 (1-2)**	0 (0-1)***	<0.001
Inflammation*	0 (0-1)	2 (2-3)**	1 (0-1)	<0.001
Total score*	1 (1-3)	8 (7-9)**	5 (4-7)	<0.001

ControlG: Control group, MTXG: Methotrexate group, MTX+BBG: methotrexate+bilberry extract group. Data expressed as median (min-max) and p values p<0.05 were regarded as statiscally significant.

* Median (minimum-maximum)

** p<0.05 compared with ControlG according to post-hoc Dunn's test.

*** p<0.05 compared with MTXG according to post-hoc Dunn's test.

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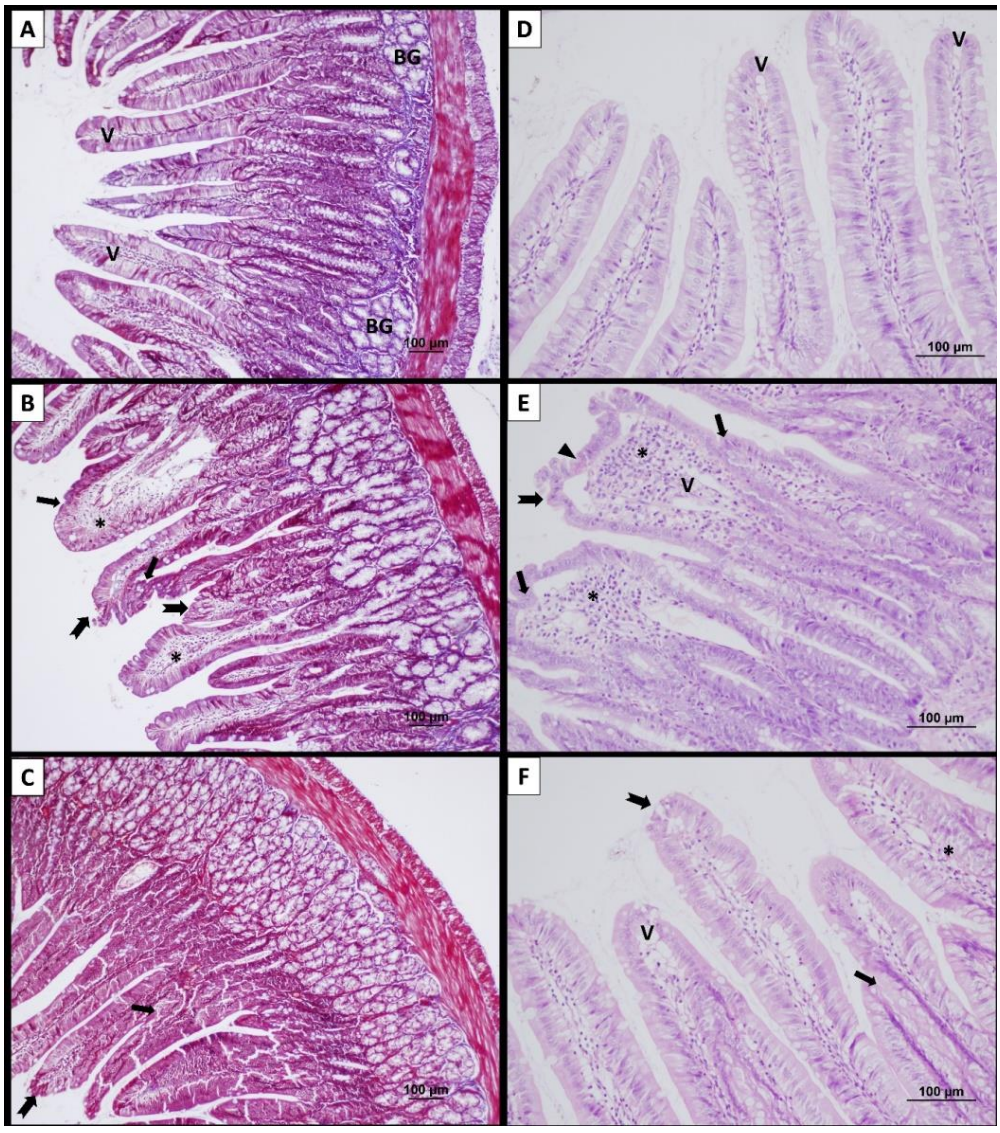

Figure 1: ControlG (A and D); Normal villi (V) covered by a regular epithelium and Brunner's glands (BG) in the tunica submucosa. MTXG: (B and E); villous fusion (arrow), hemorrhage (arrowhead), inflammatory cell infiltration (star), and degeneration in the villus epithelium (notched arrow). MTX+BBG (C and F); the villus (V) structure is much improved, and decreases can be seen in villous fusion (arrow), hemorrhage, inflammatory cell infiltration (star), and degeneration in the villus epithelium. (A, B and C; Masson's trichrome x100 and D, E and F; HE x200). ControlG: Control group, MTXG: methotrexate group, MTX+BBG: Methotrexate+bilberry extract group, HE:Hematoxylin-eosin.

DISCUSSION

Inflammation of and damage to the intestinal mucosa, some of the side-effects of chemotherapy in the gastrointestinal tract, not only reduce tolerance to treatment, but can also lead to several undesirable outcomes, including mortality (20). Similarly to various other chemotherapeutics, MTX can also produce toxic effects in the gastrointestinal system. Various studies have shown that MTX leads to pathologies including shortening of intestinal villi, fusion, edema, hemorrhage, inflammation, epithelial degeneration, and impairment of the mucosal barrier (7,12,21).

Consistent with the previous literature, we also detected marked villous fusion, epithelial degeneration, hemorrhage, and inflammatory cell infiltration in MTXG in the present study (21,22). The apoptosis marker caspase-3 also increased in MTXG, again in agreement with the literature (4,9). In addition, and consistent with other studies, TOS and OSI indicating oxidative stress increased in MTXG, while TAS, showing tissue antioxidants, decreased (4). The increase

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in MDA, which shows the damage caused by ROS in membrane lipids, as well as in 8-OHdG, showing the damage caused by DNA (23), in MTXG, also suggested that MTX causes oxidative stress in intestinal tissue. Oxidative stress is one of the irreducible factors inducing apoptosis in tissues (24). For these reasons, we concluded that the increase in apoptosis in MTXG might be associated with DNA damage induced by MTX, as well as to oxidative stress resulting from increased ROS induced by MTX (20). Overproduction of ROS is known to be capable of causing inflammation in addition to cell damage (25). We therefore concluded that inflammation in MTXG might also be associated with oxidative stress in the intestines caused by MTX (11,22).

Bilberry has been reported to reduce oxidative stress developing in tissues such as the kidney, ovary, and retina in various experimental models (17,26,27). In addition, Jakesevic et al. reported that BB reduced oxidative stress-related intestinal inflammation and mucosal damage resulting from ischemia-reperfusion, and Widen reported that it ameliorated gingival inflammation (28,29). Similarly in the present study, the decrease in oxidative stress indicators and inflammation MTX+BBG tissues may be associated with the powerful antioxidant and anti-inflammatory properties of BB (15). The decreases in caspase-3, which was elevated in MTXG, and in histologically detected mucosal damage in MTX+BBG can also be attributed to BB reducing oxidative stress.

In conclusion, MTX can lead to tissue damage in the small intestine by inducing oxidative stress inflammation, and apoptosis. Bilberry can reduce intestinal damage caused by MTX through its antioxidant, anti-inflammatory, and antiapoptotic mechanisms. Although further studies are now needed its efficacy and effect mechanisms, due to its ease of oral use BB may represent an oral supplement capable of use in preventing the toxic intestinal effects of MTX.

*Conflict of Interest***:** The authors have indicated no conflicts of interest regarding the content of this article.

Researchers' Contribution Rate Statement: Concept/Design: GK, GB, SK and SD; Analysis/Interpretation: GK, GB, NTA, SD and YA; Data Collection: GK, GB, SK, NTA and SD Writer: GK, GB, SK and SD; Critical Review: GK, SK, SD and YA; Approver: GK, SK, GB, NTA, SD and YA.

Support and Acknowledgment: No financial support was received from any institution or person.

Ethics Committee Approval: All experimental techniques utilized in this work were reviewed and approved by Karadeniz Technical University Animal Experiments Local Ethics Committee, Türkiye (number 2022/50).

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