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Research Article / Araştırma Makalesi

# Therapeutic Potential of Morus Nigra on 5-Fluorouracil-İnduced Gastrointestinal Mucositis in Rats

## Morus Nigra'nın Sıçanlarda 5-Florourasil ile Oluşturulmuş Gastrointestinal Mukozite Karşı Olası Tedavi Ediciliği

[1]Semra Yigitaslan, [2]Ayse Ozkaraman, [3]Ayfer Acikgoz, [2]Guler Balci Alparslan, [1]Cigdem Toprak, [5]Fatih Goger, [6]Erhan Sahin

[1]Department of Pharmacology, School of Medicine, Eskisehir Osmangazi University, Eskisehir, Turkey
[2] Department of Pediatry, School of Health Sciences, Eskisehir Osmangazi University, Eskisehir, Turkey
[3]Department of Internal Medicine, School of Health Sciences, Eskisehir Osmangazi University, Eskisehir, Turkey
[4]Yunus Emre Vocational School, Department of Pharmacy, Program in Pharmacy Services. & Faculty of [5]Pharmacy, Department of Pharmacognosy, Anadolu University, Eskisehir, Turkey
[6]Department of Histology and Embryology, School of Medicine, Eskisehir Osmangazi University, Eskisehir, Turkey

**Abstract:** To investigate the therapeutic potential of Morus Nigra on the mucositis of digestive system induced via 5-fluorouracil in rats. A total of 26 rats were divided into 4 groups as control (C), mucositis (M), Morus nigra extract-1(MN-1) and Morus nigra extract-2 (MN-2). Mucositis was induced by intraperitoneal injection of 60 mg/kg 5-fluorouracil (5-FU). The antioxidant enzyme levels in blood samples were analysed by ELISA. Histological analysis and immunohistochemical staining for NF-kB and TNF-α were done. Increased oxidative markers were found in mucositis group and increased antioxidative status was found in MN-1 and MN-2 groups. All histochemical analyses suggested that 5-FU causes intestinal degeneration; the MN-1 extract reduced this degeneration but did not completely treat it. The therapeutic potential of MN-2 extract was lower than the MN-1. Results of the present study suggest that, though partly, MN-1 extract in particular may have biochemically and histologically positive effects against experimental mucositis induced by 5-FU in rats.

**Keywords:** Mucositis; Rat; Morus Nigra; 5-Fluorouracil; Oxidative stress

**Özet:** Bu çalışmanın amacı sıçan gastrointestinal mukozasında 5-florourasil ile oluşturulmuş mukozit üzerine Morus nigra ekstresinin olası tedavi edici etkisini araştırmaktır. Toplam 26 adet sıçan eşit olarak kontrol, mukozit, mukozit+morus nigra ekstre 1 (MN-1) ve mukozit + morus nigra ekstre 2 (MN-2) olarak bölünmüştür. Mukozit sıçanlarda 60 mg/kg 5-fluorourasilin (5-FU) intraperitoneal olarak enjeksiyonuyla oluşturulmuştur. ELISA ile kandaki oksidan ve antioksidan seviyeleri ölçüldü. Histokimyasal ve immunohistokimyasal boyamalar (NF-kB ve TNF-α antikorları için) yapıldı. Mukozit grubunda artmış oksidatif stres belirteçleri görülürken Morus nigra ekstresi verilen gruplarda antioksidan belirteçlerin arttığı saptandı. Histokimyasal olarak 5-FU'nun mukozit oluşturduğu ve MN-1 ekstresinin tam olarak olmasa da tedavi edici olduğu gösterildi. MN-1 ekstresi MN-2 ekstresine göre daha iyi bir tedavi edici ajan olduğu belirlendi. Sonuç olarak bu çalışmada 5-FU ile oluşturulmuş mukozite karşı MN-1 ekstresinin kısmi olarak tedavi edici olduğu biyokimyasal ve histopatolojik olarak gösterildi.

**Anahtar Kelimeler**: Mukozit, Sıçan,Morus nigra,5-florourasil

***ORCID ID of the authors:*** S.Y 0000-0001-6722-2394, A .Ö 0000-0002-0507-4100, A. A 0000-0002-7507-3824, G.B.A 0000-0003-3734-3843, Ç.T 0000-0003-3718-5195, F.G 0000-0002-9665-0256, E.Ş 0000-0003-2152-0542

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**Correspondence**: **Semra YİĞİTASLAN** - Department of Pharmacology, School of Medicine, Eskisehir Osmangazi University, Eskisehir, Turkey
e-mail: drscelebi@yahoo.com

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## 1. Introduction

Cancer treatment deteriorates the mucous membrane integrity in oral cavity, pharynx, larynx, oesophagus, and other parts of gastrointestinal tract, and resulting in mucositis (1-3). The incidence of cancer treatment-induced mucositis is 85-99% in radiotherapy-treated head and neck cancer patients, 5-40% in patients with solid tumors, >50% in paediatric oncology patients and 77.9% after bone marrow transplantation (4-8).

Following chemotherapy, DNA damage as well as proinflammatory and inflammatory cytokine release and monocyte and macrophage increase are seen. After this stage, widespread and marked ulceration from epithelium to the submucosa layer, nerve damage and pain occur (2, 9, 10).

Treatment methods available for the management of oral and gastrointestinal mucositis are increasing in number day by day. While previous clinic studies report that the application of oral care protocols (ice treatment, topical analgesics, allopurinol, vitamin E, immunoglobulins) may be effective, some animal studies suggest use of several agents such as palifermin, velafermin, IL-11 and epidermal growth factor (EGF) (11-13).

Other potential treatment methods for mucositis include alternative and natural therapies. Being one of these therapies, black mulberry (Morus Nigra) has antioxidant, antimutagenic and anticarcinogenic characteristics because of containing flavonoid, anthocyanin, and carotenoid (14-16). No toxicological effect of Morus Nigra extract has been found in previous studies; however, the oral application of Morus Nigra has been reported to substantially increase the antioxidant capacity in rats (14, 16).

Present experimental study investigated the therapeutic potential of Morus Nigra on the mucositis of digestive system induced by the systemic administration of 5-fluorouracil in rats.

## 2. Materials and Methods

The study was carried out as an experimental animal model. All animal experiments were conducted according to the guidelines for the care and use of laboratory animals at Eskisehir Osmangazi University and consent was obtained from the local ethics committee (2015-372/1).

### *Animals*

A total of 27 rats were divided into 4 groups: Control group (C; n=6), Mucositis group (M; n=7), Morus nigra extract-1 group (MN-1; n=7) and Morus nigra extract-2 group (MN-2; n=7). However, because one of the rats from MN-2 group died during the experimental stage, the data have been collected from a total of 26 rats.

### *Preparation of Morus Nigra extracts*

Six kilograms of ripe *Morus Nigra* was picked at June 2015 from an orchard at Mihalgazi town of Eskisehir and was deep-freezed for 2 months. Then, the frozen *Morus Nigra* has been extracted by using two different polarity solvents into two different extracts; *Morus Nigra*-1 extract (MN-1) and *Morus Nigra*-2 extract (MN-2).

*MN-1 extract*: After being crushed while still in icy condition, 3 kilograms of *Morus Nigra* has been poured into 6 liters of acetone containing 0.05% HCl and left for maceration for 24 hours at room temperature and then has been leached. Acetone has been removed from the filtrate under low pressure and temperature. Following these steps, 300mg/ml of pulpy aqueous extract has been obtained. The aqueous section (2 liters) has been portioned into vials and then froze (-20°C) for further use in experiments.

*MN-2 extract*: After being crushed while still in icy condition, 1.5 kilograms of *Morus Nigra* has been poured into 6 liters of 96% ethyl alcohol containing 0.05% HCl and left for maceration for 24 hours at room temperature and then has been leached. Ethyl alcohol has been removed from the filtrate under low pressure and temperature. The aqueous section (1 lt) has been portioned into vials and then froze (-20°C) for further use in experiments. 166mg/ml of pulpy aqueous extract has been obtained.

***Experimental procedure***

After being weighed, the four groups of rats received the following treatments:
*Control group (C):* Each of the animals was treated with saline by oral gavage throughout the treatment.
*Mucositis group (M*): Each of the animals in this group was treated with intraperitoneal injection of 60 mg/kg 5-FU (Koçak Farma Pharmaceuticals, Istanbul, TURKEY) every other day for a total of 11 days. It has been reported that oral and gastrointestinal mucositis will be induced from the fifth day via this method (17).
Morus Nigra-1 group (MN-1): These animals were daily treated with MN-1 extract by using a nasogastric tube from the beginning until the end of the experimental procedure and underwent the same 5-FU treatment as of those in group "M" from the first day on.
*Morus Nigra-2 group (MN-2):* These animals were daily treated with MN-2 extract by using a nasogastric tube from the beginning of the experiment until the end and underwent the same 5-FU treatment as of those in group "M" from the first day on.

***Data Collection***

Twenty-four hours (Day 11) after the last dose of the treatments, blood sample was obtained from the heart and a piece of tissue was obtained from ileal mucosa for histologic examination from each animal under general anaesthesia. The tissues were kept in 4% paraformaldehyde until being analysed.

***Oxidative stress measurement***

The antioxidant enzyme levels – SOD (Cayman Chemicals, M1, USA), catalase (Elabscience Biotechnology Co. Ltd, PRC), and glutathione peroxidase (Elabscience Biotechnology Co. Ltd, PRC) – in blood samples has been analysed by using ELISA method by complying with the manufacturer's directions.

***Histopathological analysis***

*Preparation of histological slides*: Pieces of tissues cut 1 $cm^3$ in size for light microscopic study were retained in 10% neutral formalin for 24 hours and then have been made into paraffin-embedded blocks after routine histologic processes. Sections which are 5 μm in thickness were cut out of these blocks and then put on poly-L-lysine slides for Hematoxylin-Eosin, Masson's trichrome, alcian yellow and immunohistochemical staining.
Immunohistochemical staining for NF-kB and TNF-α were also made on paraffin-embedded sections.

***Statistical analysis***

The statistical analysis was carried out via IBM SPSS software package (IBM Corp. Released 2012). All the data obtained from the groups treated with *Morus Nigra* extracts were compared with control values and those of the animals that were treated with only 5-FU. The NF-KB values were analyzed after being normalized. One-way Analysis of Variance (One-way ANOVA) was used for comparing the groups. Tukey and Tamhane tests were used as multiple comparison and the data were given as mean±SEM. The $P<0.05$ probability values were accepted as significant.

## 3. Results

While there were no significant difference between the groups in terms of bodyweight (BW) values on day 0 ($p>0.05$), there was a significant difference at the end of the study between the groups in terms of BW and the ratio of bodyweight percentage change (BW%) ($p<0.05$ and $p=0.01$, respectively) (Table 1). While the BW% was significantly lower in the mucositis group compared to control group ($p<0.001$), MN-1 group showed a significant increase in the bodyweights compared to mucositis group ($p<0.05$); however, there was no significant difference in BW% between the mucositis and MN-2 groups ($p>0.05$) (Table 1).

**Table 1.** Body weights at the beginning and end of the study and the percentage body weight change observed at the end of the study (***p<0.001 compared to the control; $^{+}$p<0.05 compared to the mucositis group).

| Groups | BW at the beginning (g) | BW at the end (g) | BW increase (BW%) |
|---|---|---|---|
| **Control** | 256.33±9.09 | 275.00±2.99 | 0.07±0.02 |
| **Mucositis** | 246.43±20.29 | 231.00±22.63 | -0.06±0.03*** |
| **MN-1** | 257.43±10.05 | 273.86±30.06 | 0.06±0.09$^{+}$ |
| **MN-2** | 243.50±5.61 | 224,17±32.71 | -0.08±0.12 |

Superoxide dismutase activity, catalase, glutathione peroxidase (GPx) and NF-kB levels measured in blood samples are shown in Figures 1-4. According to these findings, the SOD activity in mucositis group was significantly lower than that of control group (p<0.01), whereas it was significantly increased in groups treated with MN-1 and MN-2 extracts (p<0.05) (Figure 1). On the other hand, although catalase and GPx values were significantly lower in the mucositis group when compared to the control group (p<0.05 and p=0.01, respectively), MN-2 extract significantly increased catalase and GPx values compared to the mucositis (p<0.05 for both). Whereas, MN-1 extract significantly increased GPx levels (p<0.05), but it increased the catalase levels only slightly (p>0.05) (Figure 2 and 3).

NF-kB levels significantly increased in the mucositis group compared to the control group (p<0.01), whereas it has significantly decreased after treatment with MN-1 extract (p<0.05). Although not being statistically significant, MN-2 extract also decreased the NF-kB levels (p>0.05) (Figure 4).

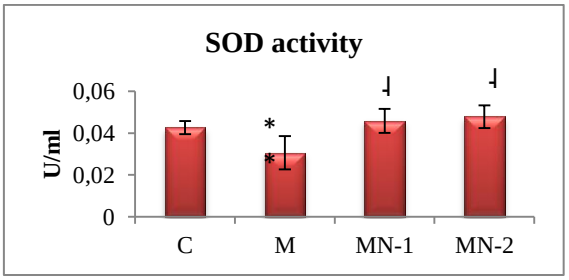


**Figure 1.** Superoxide dismutase activity in blood samples. C: control group; M: mucositis group; MN-1: Morus nigra treated group-1; MN-2 Morus nigra treated group-2. **: different from control group p<0.01); ɬ: different from mucositis group (p<0.05).

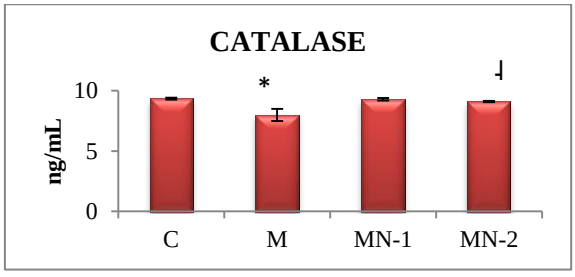


**Figure 2.** Catalase level in blood samples. C: control group; M: mucositis group; MN-1: Morus nigra treated group-1; MN-2 Morus nigra treated group-2. *: different from control group (p<0.05); ɬ: different from mucositis group (p<0.05).

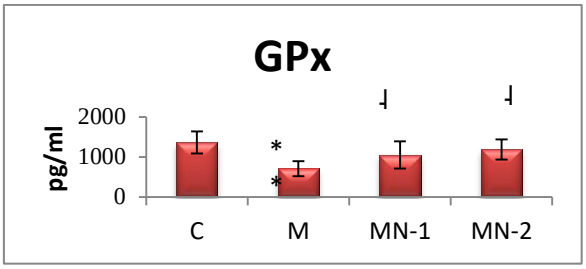


**Figure 3.** Glutathione peroxidase level in blood samples. C: control group; M: mucositis group; MN-1: Morus nigra treated group-1; MN-2 Morus nigra treated group-2. **: different from control group (p<0.01); ǂ: different from mucositis group (p<0.05)

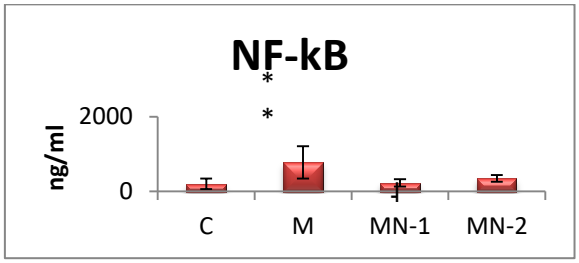


**Figure 4.** NF-kB level in blood samples. C: control group; M: mucositis group; MN-1: Morus nigra treated group-1; MN-2 Morus nigra treated group-2. *: different from control group (*: p<0.05; **: p<0.01; ***: p<0.001); ǂ: different from mucositis group (ǂ: p<0.05; ǂ ǂ: p<0.01; ǂ ǂ ǂ: p<0.001).

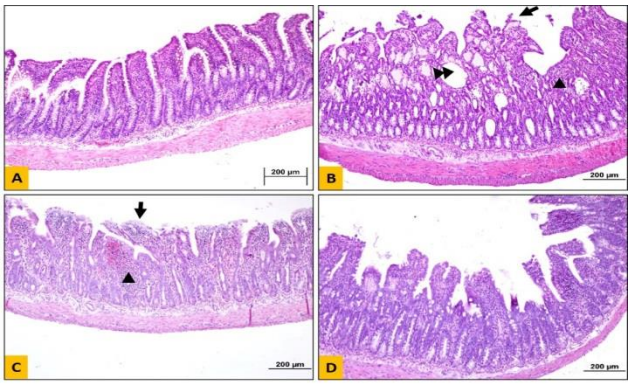


**Figure 5.** Hematoxylin eosin staining of small intestine. A: Control group shows normal small intestine histological structure. B: Mucositis group shows mononuclear cell infiltration (one arrow head), epithelial degeneration (arrow) and mucosal edema (Two arrow head). C: Mucosal degeneration and mononuclear cell infiltration still existed in the animals treated with MN-2 extract, but it was not much as observed in the mucositis group. D: MN-1 extract reduced the mucosal degeneration caused by 5-FU, resulting in a histological structure close to that found in the control group. The bars are all 200μm.

Histopathologic examinations were carried out through hematoxylin-eosin, Masson's trichrome, and alcian yellow staining methods. In 5-FU treated rats it was found that epithelial degeneration is obvious and existent villus are connected to each other. Besides, there was an oedema in lamina propria and extensive infiltration of mononuclear cells. When the treatment groups were analysed, it was found that although not being a complete healing, MN-1 extract reduced the mucosal degeneration caused by 5-FU, resulting in a histological structure close to that found in the control group. Mucosal degeneration and mononuclear cell infiltration still existed in the animals treated with MN-2 extract, but it was not much as observed in the mucositis group (Figure 5).

All groups have similar histological structure in terms of connective tissue (Figure 6). The number of goblet cells examined by alcian yellow staining was found to be decreased in mucositis and MN-2 groups, whereas it was similar in the control and MN-1 groups (figure 7).

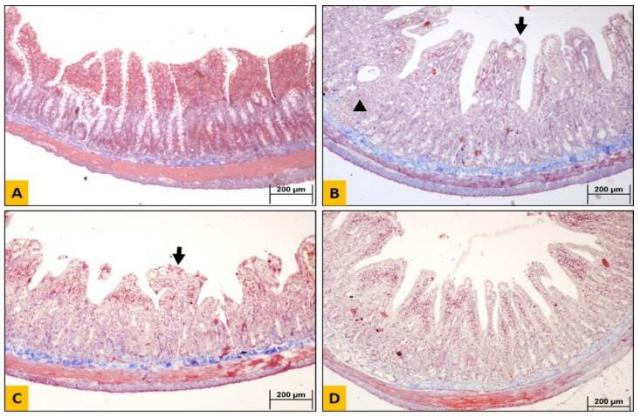


**Figure 6.** Masson's trichrome staining of small intestine. A: Control, B: Mucositis, C: MN-2 group, D: MN-1 group. All groups have similar histological structure in terms of connective tissue. Mucositis group shows mononuclear cell infiltration (one arrow head) and epithelial degeneration (arrow). The bars are all 200µm.

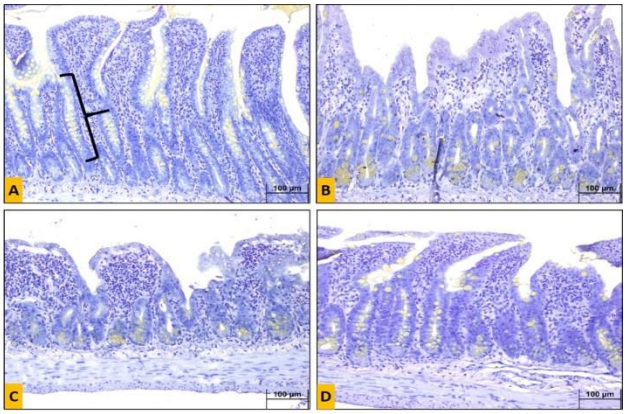


**Figure 7.** Alcian yellow staining of small intestine. A: The crypts of Lieberkuhn (brackets) and goblet cells (yellow stained cells) in control group shows normal histological structure. B: Mucositis group shows decreased goblet cell counts. C: In MN-2 group goblet cells increases but not too much. D: The MN-1 extract increases goblet cells and the histological structure is close to control group. The bars are all 100µm.

NF-κB and TNF-α staining carried out by indirect immunohistochemical method showed similar results; there has been a higher level of immunoreactivity for both antibodies in the mucositis group, this reactivity decreased in MN-1 group while a small amount of reactivity was still present in MN-2 group (figure 8 and 9).

All histochemical and immunohistochemical analyses suggest that 5-FU causes intestinal degeneration; and the MN-1 extract reduced this degeneration but was not completely treated it, while the therapeutic effect of MN-2 extract was lower than that of MN-1.

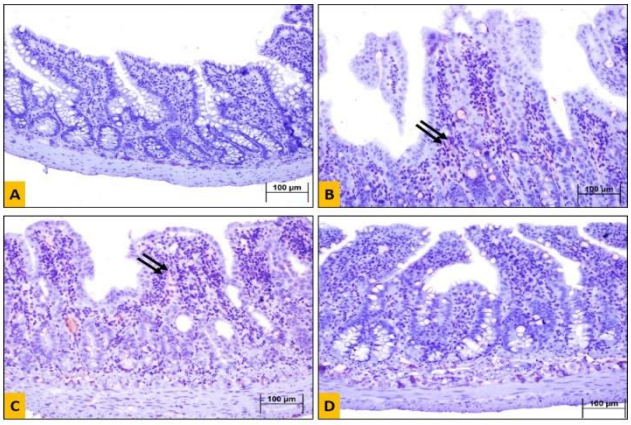


**Figure 8.** NF-κB staining of small intestine. A: Control group shows no immunostaining. B: Mucositis group shows high immunostaining (Double arrow) in lamina propria. C: MN-2 group shows decreased immnustaining (mild) but not too much, its still close to mucositis group. D: MN-1 group shows decreased immunostaining, according to staining this group close to control group. The bars are all 100µm.

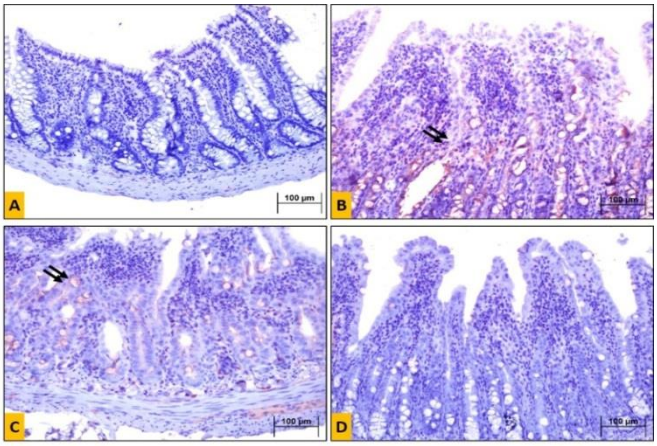


**Figure 9.** TNF-α staining of small intestine. A: Control group shows no immunostaining. B: Mucositis group shows high immunostaining (Double arrow) in lamina propria. C: MN-2 group shows decreased immnustaining but not too much, its still close to mucositis group. D: MN-1 group shows decreased immunostaining, according to staining this group close to control group. The bars are all 100µm.

## 4. Discussion

The possible therapeutic effects of two different *Morus Nigra* extracts (MN-1 and MN-2) was evaluated in this study in which a mucositis model was experimentally induced in rats by using 5-FU and it has been observed that MN-1 extract in particular has biochemically and histologically positive effects on gastrointestinal mucositis. Although MN-2 extract has also shown some positive effects, the healing was insufficient in terms of histological findings.

Oral and gastrointestinal mucositis is an adverse effect of cancer chemotherapy and the treatment methods available currently only aim eliminating the symptoms and preventing the development of infections. In recent years, a number of agents (17-20) such as interleukin receptor antagonists (11), insulin-like growth factor 1 (21), minocycline (22), uridine phosphorylase-1 inhibitors (23) and taurine (24) has been studied in order to increase the life quality and prevent and/or eliminate this toxic effect completely.

The chemotherapy-induced gastrointestinal mucositis includes complex biological processes mediated by inflammatory

cytokines and reactive oxygen species where oxidative stress indices and TNFα and NF-KB plays a vital role (25, 26).

In the present study, SOD activity, catalase, and glutathione peroxidase (GPx) levels which are biomarkers of oxidative stress were measured in blood samples of the animals and it has been observed that the oxidative stress level increased in untreated mucositis group, whereas the oxidative stress decreased in treated groups, which was more significant particularly in the group treated with MN-1 extract. *Morus Nigra*, also named as black mulberry, contains lots of biologically active agents such as amylose, flavonoids and alkaloids (15, 16). Among these, flavonoids have a variety of physiological effects such as being antioxidant, antimicrobial, anti-inflammatory, anticancer, and anti-radiation (27, 28). Many other studies have also reported as similar to the results of this study that *Morus Nigra* extracts have a strong antioxidant effect (27-30). We have used two different extracts of black mulberry and have found out that they have different effects on chemotherapy-induced gastrointestinal mucositis. This difference between the two extracts may be related to the difference between flavonoid contents, thus to their antioxidant capacities.

Reactive oxygen species produced after chemotherapy-induced gastrointestinal mucositis causes the activation of NF-kB transcription factor and the release of proinflammatory cytokines, especially TNF-α (25). If proinflammatory cytokines are not eliminated from the body, they continue to damage the tissue. Nonsteroidal and steroidal anti-inflammatory drugs (NSAIDs and SAIDs), even if having anti-inflammatory capacity, could not be used in the treatment of mucositis because of gastrointestinal adverse effects and this issue brings up the possibility of usage of alternative and natural compounds having lesser adverse effects. It is reported that the black mulberry fruit used in various studies on animals (27) and also in humans during pharyngitis (31) and arthritis (32) have a strong anti-inflammatory effect.

Inflammation comprises many pathological cases such as arachidonic acid pathway, cytokines, nitric oxide (NO), mitogen activated protein kinase (MAPK) and NF-kB (33). Flavonoids are reported as multi-targeted therapeutic agent targeting more than one of these pathways. As a matter of fact, in the study carried out by Chen et al (27) investigating the anti-inflammatory and antinociceptive activity of black mulberry, it has been found that black mulberry is rich in flavonoids and contains two types of anthocyanin (cyanidin 3-glucoside and cyanidin 3-rutinoside). It has been reported in a study that total flavonoid content of black mulberry inhibits cytokines and also NO level at RAW 264.7 cell line and in another study that a herbal flavone suppresses COX-2 production (34).

In accordance with these findings, we have found out that the black mulberry, particularly MN-1 extract, shows an anti-inflammatory effect by reducing increased TNF-α and NF-ƙB levels in mucositis-induced animals. Similarly, we have observed during histological evaluation that mucosal degeneration and inflammatory cell infiltration induced by 5-FU decreased, especially in the group treated with MN-1 extract.

Mucositis occurs by molecular and cellular events related to all components (epithelium and ligament) of the mucosa and according to the current classification, there are 5 stages in the pathogenesis: initiation, upregulation and damage response, signal amplification, ulceration, and finally healing stages (25). Many anticancer agents like 5-fluorourasil induce mucosal damage due to the inflammatory response by increasing ROS release in both normal tissue and cancer cells (18, 35). Similarly, the the severe mucosal degeneration found in the mucositis group partially healed in animals treated with MN extracts, suggesting that these two extracts of black mulberry may have ability to scavenge ROS.

The therapeutic potential of two different *Morus Nigra* extracts supposed to have antioxidant and anti-inflammatory effects (MN-1 and MN-2) was evaluated in this study in which gastrointestinal mucositis was experimentally induced in rats by using 5-FU and it has been observed that, though partly, MN-1 extract in particular may have biochemically and histologically positive effects against mucositis. However, with

different administration routes, further studies evaluating the preventive and therapeutic activity of the black mulberry fruit against gastrointestinal mucositis and also other possible therapeutic effect mechanisms of black mulberry extracts on mucositis will help to evaluate these natural and alternative agents with minimal adverse effects to be used solely or with other agents before and/or during chemotherapy and/or radiotherapy.

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