Annals of Clinical and Analytical Medicine
Original Research

Methylene blue ameliorates colonic injury in acetic acid-induced colitis model

Methylene blue and experimental colitis

Yusuf Tanrıkulu[1], Ceren Sen Tanrıkulu[2], Fahriye Kılınç[3], Murat Can[4], Furuzan Köktürk[4], Süleyman Kargın[1], Gökhan Yılmaz[5]
[1] Department of General Surgery, Faculty of Medicine, KTO Karatay University, Konya
[2] Department of Emergency Medicine, Health Sciences University, Konya Education and Research Hospital, Konya
[3] Department of Pathology, Faculty of Medicine, Necmettin Erbakan University, Konya
[4] Department of Biochemistry, Faculty of Medicine, Bulent Ecevit University, Zonguldak
[5] Department of General Surgery, Faculty of Medicine, Medipol University, Istanbul, Turkey

Abstract
Aim: Inflammatory bowel disease (IBD) is a serious health problem affecting the quality of life. IBDs caused by various pathophysiological mechanisms, including genetic, immunological, inflammatory, and environmental factors. Although it has been widely investigated, treatment of IBDs remains a difficult clinical problem. We aimed to investigate the healing of mucosal damage and anti-inflammatory effects of methylene blue (MB) in experimental colitis model.
Material and Methods: Twenty-eight rats were randomly divided into four equal groups: group 1 (Sham), group 2 (control), group 3 (topical treatment), and group 4 (topical and systemic treatment). In groups 2, 3, and 4, acetic acid-induced colitis model was created. Normal saline to group 2, topical MB to group 3, and topical and systemic MB to group 4 were given.
Results: The levels of hydroxyproline were lower in the treatment groups than in the control group (p=0.017 for group 3 and p=0.004 for group 4). There was no difference between groups according to the TNF- α levels. The results of macroscopic scores were significantly lower in treatment groups (group 3-4) than in the control group (group 2) (p=0.005 for group 3 and p=0.002 for group 4). There was a significant difference between the treatment groups and the control group according to the epithelial loss and inflammatory infiltrate (p=0.022 for group 3, p=0.026 for group 4 for both histopathological values).
Discussion: MB ameliorates colonic injury in acetic acid-induced colitis model. This effect of MB was observed both locally and with local + systemic administration.

Keywords
Acetic Acid, Colitis, Inflammatory Bowel Disease, Methylene Blue, TNF-α

DOI: 10.4328/ACAM.20889 Received: 2021-10-09 Accepted: 2021-11-11 Published Online: 2021-11-13 Printed: 2022-02-01 Ann Clin Anal Med 2022;13(2):146-150
Corresponding Author: Yusuf Tanrıkulu, Department of General Surgery, Faculty of Medicine, KTO Karatay University, 42020, Karatay, Konya, Turkey.
E-mail: drtanrikulu@hotmail.com P: +90 505 657 97 09
Corresponding Author ORCID ID: https://orcid.org/0000-0001-8432-2119

Introduction

Inflammatory bowel disease (IBD) - Crohn's disease (CD) and ulcerative colitis (UC), a systemic disorder involving primarily gastrointestinal tract, may be affecting the quality of life of patient promptly. IBD is caused by various pathophysiological mechanisms such as genetic, immunological and environmental factors. Patients with IBD mostly admit to the health care centers with abdominal pain, diarrhea, with or without blood [1,2]. Sulfasalazine, mesalazine, and steroids are the conventional medications for IBDs. In cases where patients do not respond to these medications, immunosuppressive agents such as azathioprine and cyclosporin that have significant side effects are used [3].

Many experimental colitis models have been developed for IBDs. The most widely used models are using chemical agents such as trinitrobenzene sulfonic acid (TNBS), acetic acid, dinitrochlorobenzene, dextran sodium sulfate, peptidoglycan, and polysaccharide. These agents, used to create the colitis model can be administered through the, through drinking water or gastric lavage, or via multiple intramural injections [4]. Acetic acid (AA) administration is one of the models for experimental colitis in rats [5].

In the pathogenesis of IBDs, both the natural (macrophages and neutrophils) and acquired (T and B lymphocytes) immune responses and the loss of tolerance against the bacteria in the enteric flora play key roles. The main change seen in the natural immune response is increased number of macrophages and dendritic cells activation in the lamina propria [7].

Hydroxyproline is an important constituent of the major structural protein, and plays a key role in the synthesis and stability of collagen. A normal and stable cellular structure of the extracellular matrix is a sign of complete wound healing process after tissue injury. Increased hydroxyproline content in granulation tissue is the indicator of increased collagen turnover, which indicates better maturation and proliferation of collagen during wound healing [7].

Methylene blue [MB], identified by Ehrlich in 1981, is widely used for its anti-adhesive and anti-inflammatory features. The most common medical applications of MB are refractory distributive shock, carbon monoxide poisoning, methemoglobinemia, corrosive esophageal injury, and encephalopathy [8]. In inflammation, although the main effect of MB is still not fully clarified, it shows its effect by inhibiting nitric oxide synthesis, increasing the antioxidant capacity, and reducing fibrinolytic activity [9,10]. We aimed to investigate the anti-inflammatory effects of MB in experimental colitis model in rats.

Material and Methods

Study Design and Animals

The experimental procedures were made based on the Turkish Guidelines for Laboratory Animals after obtaining approval from the local Ethics Committee for Animal studies at Bülent Ecevit University (Date/Number: 2014/13). Twenty-eight adult male Wistar-Albino rats, weighing 260±30 g, were used in the study. The rats were individually maintained at normal room temperature (21±1 °C) and fed with a standard diet and water. The animals were deprived of food for 12 hours before anesthesia. However, no water restriction was performed 2 hours before anesthesia. No enteral or parenteral antibiotics were administered at any time.

Induction of Colitis

The rats were anaesthetized with intramuscular ketamine (50 mg/kg) and xylazine (6 mg/kg). After inducing anesthesia, 1 mL of acetic acid (AA) (5%) in isotonic saline was instilled using a soft 6-Fr pediatric catheter inserted into the anus up to 6-8 cm. Eventually, after 15-20 seconds of exposure, the fluid was withdrawn. Before the withdrawal of the catheter, 2 mL of air was applied to distribute AA in the colon. In the treatment groups, the MB was administered one hour after induction. This experimental model has been shown to have similar histopathological features in human colitis [11].

Experimental Groups

Twenty-eight rats were randomly divided into four equal groups. The colitis model was produced in all groups except the sham group.

Group 1 (Sham): After each rat was rectally given saline, 1 mL of saline was intraperitoneally applied.

Group 2 (Control): After the colitis model occurred, 1 mL of saline was intrarectally applied. No treatment was given.

Group 3 (Topical treatment): One hour after the colitis model was occurred, a dose of 2 mL of 1% MB (Blumet®, Defarma, Tekirdağ, Turkey) was rectally applied. The rats were incubated for 10 min in a Trendelenburg position to provide sufficient mucosal contact with the drug. Subsequently, 1 mL of saline was intraperitoneally administered.

Group 4 (Topical+Systemic treatment): One hour after the colitis model occurred, 2 mL/kg of 1% MB was rectally administered. The rats were kept in the Trendelenburg position for 10 minutes to ensure adequate drug-mucosal interaction. Subsequently, a 0.1 mL dose of 1% MB was administered intraperitoneally to each rat as a single daily dose for ten days.

On the tenth day, the abdomen and thorax were opened through a midline incision under anesthesia. Blood samples were taken by cardiac puncture method for biochemical analysis, and the rats were sacrificed by the same method. Later, the distal 8 cm of the colon was excised for histopathological evaluation.

Histopathological Evaluation

Mucosal injury was evaluated macroscopically using the grading scale of Morris et al. just after being washed with saline solution. According to this scoring system, no damage is calculated as 0 points, localized hyperemia, but no ulcers are 1 point, linear ulcers with no significant inflammation are 2 points, linear ulcer with inflammation at one site is 3 point, two or more sites of ulceration and/or inflammation are 4 point and two or more major sites of inflammation and ulceration or one more site of inflammation and ulceration extending >1 cm along the length of the colon is 5 points [3]. A single well-

Table 1. Variables used in the classification of the inflammatory histological score [14]

Score	Epithelial loss	Inflammatory infiltrate	Integrity of crypts	Stress to goblet cells
0	None	None	Intact	Absent
1	<5%	Mild	<10%	Present
2	5-10%	Moderate	10-20%	N/A
3	>10%	Severe	>20%	N/A

experienced pathologist who was also unaware of the study design, examined each specimen. For this examination, 8- cm distal colon sections were taken. In the colon tissue samples, 10% neutral formaldehyde was used and then followed by the paraffin embedding technique. Tissue samples were obtained with a thickness of 4 µm and stained using hematoxylin-eosin (H&E), and then analyzed under a light microscope (Olympus BX53, Tokyo, Japan). The degree of inflammation of the colon was graded semi-quantitatively from 0 to 11 according to the criteria defined by Özgün et al. (Table 1) [12].

Biochemical Analysis

Blood

Blood was collected into tubes at the time of death. Blood samples were centrifuged at 1000 g for 10 minutes at 4oC to remove plasma. Aliquots of the samples were transferred into polyethylene tubes to be used in the assay of biochemical parameters and were stored at -80oC until analysis. TNF-α levels were measured in the serum using a rat TNF alpha ELISA kit (Eastbiopharm, Hangzhou) on fully automatic devices.

Tissue

All tissues were washed twice with cold saline solution, placed into glass bottles, labeled, and stored in a deep freezer (-80oC) until processing. Colon tissues were homogenized in 10 volumes of 150 mM ice-cold KCL using a glass Teflon Homogenizer (Ultra Turrax IKA T18 Basic; IKA, Wilmington, NC, USA) after cutting the tissues into small pieces with scissors (for 2 minutes at 5000 rpm). The homogenate was then centrifuged at 5000 g for 15 minutes. The supernatant was used for analysis. Later, spectrophotometry was used to detect tissue hydroxyproline concentrations, and the results were denoted as micrograms per milligram of tissue [13]. First, the samples, standards, and streptavidin-HRP were added to the well. The antibodies were labeled with an enzyme and the plate was incubated for 60 minutes at 37ºC. The plate was washed five times and chromogen solutions were added. The plate was incubated for 10 minutes at 37ºC and stop solution was added into the wells. The optical density (OD) was measured at 450 nm wavelengths with a microplate reader.

Statistical Analysis

The analysis of the results was carried out with the SPSS (Statistical Package for Social Science) for Windows 19.0 package program. Data normality was determined using the one-sample Kolmogorov-Smirnov test, and then the Kruskal-Wallis variance analysis was used for continuous variables. While the continuous variables were given as mean± standard deviation, non-continuous variables were expressed as median (min-max). The Chi-Square test was used to compare non-continuous variables. A p-value of less than 0.05 was considered statistically significant for all tests.

Results

Comparison of the groups according to the hydroxyproline and TNF-α levels is shown in Table 2. The levels of hydroxyproline were significantly lower in the treatment groups (group 3 and 4) than in the control group (group 2) (p=0.017 for group 3 and p=0.004 for group 4). The levels of hydroxyproline were lower in the sham group than that in treatment ones (no statistically significant difference). In contrast, TNF-α levels were lower in the treatment groups than that in group 2 (p=0.176).

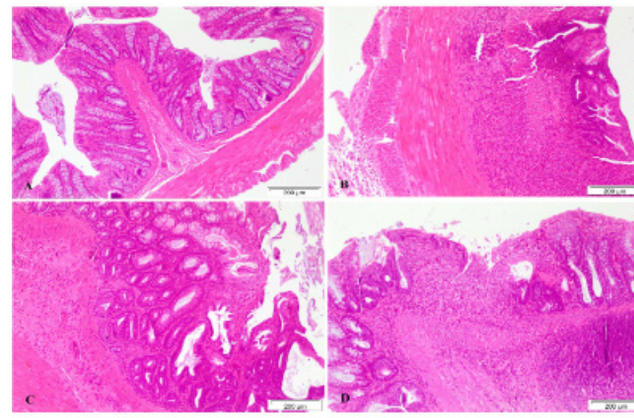

Figure 1. In histological specimens of the colon wall, the Sham group (Group 1) appears to have numerous goblet cells in the mucosal surface and crypt epithelium and sparse mononuclear cells in lamina propria. The crypts are regular and have a fine stroma (A). The control group (Group 2) contains irregularity of surface epithelium, distortion in crypts, reduction in goblet cells, expansion of the stroma between crypts and submucosa, and mixed inflammatory cells infiltrating the epithelium (B). Topically treatment group (Group 3) has a crypt distortion, goblet cell reduction in surface and crypt epithelium, inflammatory cell infiltration in the stroma (C). Topically and systemically treatment group (Group 4) appears to have some reduction and irregularity in crypts and mixed inflammatory cells in lamina propria and submucosa. Goblet cell reduction continues in crypt and surface epithelium. Stroma between crypts and slightly expanded to submucosa (D) (Hemotoksilen Eosin, x100).

Table 2. Distribution of the results of biochemical evaluation according to groups

Groups	Hydroxyproline	TNF-α
Group 1 (Sham)	3.48 ± 0.44	43.88 ± 10.06
Group 2 (Control)	5.23 ± 0.79	32.68 ± 15.67
Group 3 (Topical treatment)	3.54 ± 0.38[a]	38.57 ± 15.17
Group 4 (Topical + systemic treatment)	3.74 ± 1.25[b]	37.28 ± 14.31

TNF- α: Tumor necrosis factor alpha, [a] p=0.017, [b] p=0.004 vs. control groups

Table 3. Distribution of histopathological evaluation results according to groups

	Microscopic Scores					Macroscopic Scores
Groups	Epithelial loss	Inflammatory infiltrate	Integrity of crypts	Stress to goblet cells	Total Damage score	
Group 1	0 (0-1)	1 (0-1)	0 (0-0)	0 (0-0)	0 (0-1)	0 (0-0)
Group 2	2 (1-3)	2 (1-3)	0 (0-1)	1 (1-2)	1 (0-3)	4 (2-5)
Group 3	1 (0-2)[x]	1 (0-2)[a]	0 (0-1)	1 (0-3)	1 (0-3)	2 (1-3)[#]
Group 4	1 (0-2)[y]	1 (0-2)[b]	0 (0-0)	1 (0-1)	1 (0-2)[a]	1 (1-2)*

[x] p=0.022, [y] p=0.026, [a] p=0.022, [b] p=0.026 vs. control groups, [#] p=0.005, * p=0.002, [a] p=0.006 vs. control groups

The results of the histopathological examinations are summarized in Table 3 and Figure 1. The method described by Morris et al. [3] was used to assess macroscopic injury in the colon wall. The sham group seemed to have numerous goblet cells in the mucosal surface and crypt epithelium and sparse mononuclear cells in lamina propria, and mucosal structure was similar to the normal colon mucosa. The macroscopic scores were found to be significantly lower in the treatment groups (group 3-4) than that in the control group (group 2) (p=0.005 for group 3 and p=0.002 for group 4). Microscopic damage was evaluated according to criteria defined by Özgün et al. [14]. There was no difference between groups in terms of integrity of crypts and stress to goblet cells (p=1.000 and p=0.261, respectively). There was significant difference between the treatment groups and the control group according to the epithelial loss and inflammatory infiltrate (p=0.022 for group 3, p=0.026 for group 4 for both histopathological values). Total damage score was significantly lower in the group 4 compared to the control group (p=0.006).

Discussion

There are few studies investigating the effect of MB on the colitis model, although there are many experimental and clinical studies on colitis in the literature. Our results showed for the first time in the literature that intraperitoneal (systemic) and intrarectal (local) application of MB may reduce the severity of underlying inflammation among the rats with AA-induced colitis model.

IBDs are a chronic inflammatory disease of the gastrointestinal tract that are diagnosed based on clinical, endoscopic and histological findings. Although the combination of environmental, genetic and immunoregulatory factors has been hypothesized in the pathogenesis of the disease, the pathogenesis is still unclear. The generally accepted opinion in the pathogenesis of IBD is an inappropriate response of the mucosal immune system to the intestinal flora and other luminal antigens, which leads to inflammation [14].

Many proinflammatory cytokines (TNF, IL-2, IL-6 or INF) are upregulated in IBD. TNF is the main mediator of intestinal inflammation, and it is synthesized by many cells and activates many genes with NFKB pathway. Oxidative stress and reactive oxygen products play key roles in the development of IBD as well as in inflammatory activation. Reactive oxygen products with pro-inflammatory mediators eventually lead to mucosal ulceration and disruption. Therefore, the effect of various antioxidants and anti-inflammatory agents was investigated in experimental models of IBD [10,15].

MB has both anti-adhesive and anti-inflammatory properties. Although the main mechanism of action of MB is currently unknown, it is thought that its primary effect is associated with the nonselective NO synthesis inhibitor. Namely MB acts on both constitutive NO synthase isoforms as well as on inducible NO synthase isoform (15). Using this effect, MB has been used as an anti-adhesive in many studies [9,10]. MB also inhibits the generation of oxygen radicals as an electron acceptor. The main effect of MB in inflammation is still not fully clarified today, but this effect of MB is thought to be associated with inhibition of NO synthesis [16]. A previous study [17] suggested that MB may reduce expressions of adhesion molecules and pro-inflammatory cytokines. Dinc et al. [16] found that MB given intraperitoneally may prevent the AA-induced colitis via attenuation of oxidative stress and inflammatory pathway. In our study, even though it was not statistically significant, we showed a lower level of TNF-α in treatment groups.

In experimental colitis models induced by acetic acid, hyperemia, edema, linear ulcers and inflammation in the colon tissues indicate macroscopically inflammatory bowel disease [13]. In microscopic examinations, disruption of mucosal barrier, decrease in goblet cells and crypts, widespread cryptitis and crypt abscess, inflammatory cell growth and loss of mucus in lamina propria are defined as important histopathological findings for the diagnosis of ulcerative colitis [18]. Dinc et al. [16] found that MB given intraperitoneally reduces microscopic and macroscopic injury. Similarly, Çetinkaya et al. [19] showed that acetylcysteine has beneficial effects on acetic acid-induced colitis in rats.

Hydroxyproline is an important constituent of the major structural protein, and plays a key role in the synthesis and stability of collagen. Hydroxyproline is an important indicator of collagen accumulation. Deficiency of protein during wound healing may diminish new capillary development, fibroblast proliferation, collagen and proteoglycans synthesis and remodeling and contraction of the wound. A normal and stable cellular structure of the extracellular matrix is a sign of complete wound healing process after a tissue injury. There are numerous reasons to support the use of hydroxyproline as a biomarker of the collagen content within tissues after the wound-healing process, as it is abundantly found in collagen and plays a vital role in wound healing [7]. Sadar et al. [20] found that hydroxyproline levels increased hydroxyproline level significantly correlated with the accumulated collagen in colonic tissue after TNBS administration, and ferulic acid treatment significantly reduced the colonic hydroxyproline activity. In our study, we found that hydroxyproline levels were significantly high in the control group and significantly lower in the treatment groups in accordance with the literature (p=0.017 for topical treatment group, p=0.004 for topical + systemic treatment group).

Macroscopic and histopathological examinations are the gold standard for evaluating inflammation in the colon. Appleyard and Wallace [21] found that the total histopathological damage score was higher in colitis group compared to the saline group in the original colitis studies in which they underwent histopathological evaluation. Balaha et al. [22] found that GO ameliorated the marked macroscopic and microscopic changes of colonic mucosa in a dose-dependent manner. In our study, we found that the total damage score was significantly lower in group 4 compared to the control group (p=0.006).

Conclusion

In conclusion, we found that MB ameliorates colonic injury in acetic acid-induced colitis model. This effect of methylene blue was observed both locally and with local + systemic administration. In addition, it reduced inflammation, although not statistically significant. Therefore, due to the clinic and pharmacologic properties of MB, it can be used to treat the IBD. However, it is obvious that further and comprehensive clinical studies are needed.

Limitations

Since this study was experimental, the number of cases was limited. Due to restriction in the number of animals, treatment results for 10 days for both group 3 and group 4 could not be evaluated.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

Funding: *None*

Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

References

1. Safarpour AR, Hosseini SV, Mehrabani D. Epidemiology of inflammatory bowel diseases in iran and Asia; a mini review. Iran J Med Sci. 2013;38(Suppl. 2):S140-9.
2. Fiocchi C. Intestinal inflammation: a complex interplay of immune and nonimmune cell interactions. Am J Physiol. 1997;273(4):G769-75.
3. Morris GP, Beck PL, Herridge MS, Depew WT, Szewczuk MR, Wallace JL. Hapten-induced model of chronic inflammation and ulceration in the rat colon. Gastroenterology. 1989;96(3):795-803.
4. Rosenberg LN, Peppercorn MA. Efficacy and safety of drugs for ulcerative colitis. Expert Opin Drug Saf. 2010;9(4):573-92.
5. Pereira C, Grácio D, Teixeira JP, Magro F. Oxidative Stress and DNA Damage: Implications in Inflammatory Bowel Disease. Inflamm Bowel Dis. 2015;21(10):2403-17.
6. Sartor RB, Hoentjen F.. Mechanisms of Bacterial-Induced Experimental Colitis. In: Mestecky J, McGhee JR, Strober W, editors. Mucosal Immunology, 3rd ed.USA: Academic Press 2005,p.681-702.
7. Yun HM, Ban JO, Park KR, Lee CK, Jeong HS, Han SB, et al. Potential therapeutic effects of functionally active compounds isolated from garlic. Pharmacol Therapeut. 2014;142(2):183–95.
8. Wainwright M, Crossley KB. Methylene Blue--a therapeutic dye for all seasons? J Chemother. 2002;14(5):431-43.
9. Dinc S, Ozaslan C, Kuru B, Karaca S, Ustun H, Alagol H, et al. Methylene blue prevents surgery-induced peritoneal adhesions but impairs the early phase of anastomotic wound healing. Can J Surg. 2006;49(5):321-8.
10. Kalaycı MU, Eroğlu HE, Kubilay D, Soylu A, Sancak B, Uğurluoğlu C, et al. The effects of methylene blue on adhesion formation in a rat model of experimental peritonitis. Ulus Travma Acil Cerrahi Derg. 2011;17(3):205-9.
11. Randhawa PK, Singh K, Singh N, Jaggi AS. A review on chemical-induced inflammatory bowel disease models in rodents. Korean J Physiol Pharmacol. 2014;18(4):279-88.
12. Özgün E, Sayılan Özgün G, Eskiocak S, Yalçın Ö, Süer Gökmen S. Deneysel kolitte L-karnitinin serum paraoksonaz, arilesteraz ve laktonaz aktivitelerine ve oksidatif duruma etkisi (Effect of L-carnitine on serum paraoxonase, arylesterase and lactonase activities and oxidative status in experimental colitis). Türk Biyokimya Dergisi/ Turkish Journal of Biochemistry. 2013;38(2):145-53.
13. Bergman R, Loxley R. Two improved and simplified methods for the spectrophotometric determination of OH-proline. Anal Chem. 1963;35(12):1961–5.
14. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. Nature. 2007;448(7152):427-34.
15. Mayer B, Brunner F, Schmidt K. Inhibition of nitric oxide synthesis by methylene blue. Biochem Pharmacol. 1993;45(2):367-74.
16. Dinc S, Caydere M, Akgul G, Yenidogan E, Hücümenoglu S, Rajesh M. Methylene Blue inhibits the inflammatory process of the acetic acid-induced colitis in the rat colonic mucosa. Int Surg. 2015; DOI: 10.9738/INTSURG-D-15-00118.1.
17. Werner I, Guo F, Stock UA, Lupinski M, Meybohm P, Moritz A, et al. Methylene blue modulates adhesion molecule expression on microvascular endothelial cells. Inflamm Res. 2014;63(8):649–56.
18. Cross SS, Harrison RF. Discriminant histological features in the diagnosis of chronic idiopathic inflammatory bowel disease: analysis of a large dataset by a novel data visualisation technique. J Clin Pathol. 2002;55(1):51-7.
19. Cetinkaya A, Bulbuloglu E, Kurutas EB, Ciralik H, Kantarceken B, Buyukbese MA. Beneficial effects of N-acetylcysteine on acetic acid-induced colitis in rats. Tohoku J Exp Med. 2005;206(2):131-9.
20. Sadar SS, Vyawahare NS, Bodhankar SL. Ferulic acid ameliorates TNBS-induced ulcerative colitis through modulation of cytokines, oxidative stress, iNOs, COX-2, and apoptosis in laboratory rats. EXCLI J. 2016;15:482-99.
21. Appleyard CB, Wallace JL. Reactivation of hapten-induced colitis and its prevention by anti-inflammatory drugs. Am J Physiol. 1995;269(1 Pt 1):119-25.
22. Balaha M, Kandeel S, Elwan W. Garlic oil inhibits dextran sodium sulfate-induced ulcerative colitis in rats. Life Sci. 2016;146:40-51.

How to cite this article:
Yusuf Tanrıkulu, Ceren Sen Tanrıkulu, Fahriye Kılınç, Murat Can, Furuzan Köktürk, Süleyman Kargın, Gökhan Yılmaz. *Methylene blue ameliorates colonic injury in acetic acid-induced colitis model. Ann Clin Anal Med 2022;13(2):146-150*