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Ameliorative effects of the flavonoid fustin in a rat model of trinitrobenzensulfonic acid-induced colitis

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Abstract. Trinitrobenzene sulfonic acid (TNBS)-induced colitis is a widely used animal model that mimics the signs and symptoms of inflammatory bowel disease. Fustin is a flavonoid found in *Cotinus coggygria*.
The aim of the present study was to investigate the effect of fustin isolated from *Cotinus coggygria* heartwood in a rat model of TNBS-induced colitis.
In this experiment, 30 male Wistar rats were used, allocated to three groups: Control, TNBS and TNBS+F10. Colitis was induced by rectal application of TNBS. After the induction of colitis, fustin at a dose of 10 mg/kg was administered orally to TNBS+F10 group in the course of 8 days. Severity of colitis, oxidative stress and inflammation were assessed by macroscopic, histopathological, immunohistochemical and biochemical analyses.
Rats from TNBS group demonstrated severe colonic damage. Fustin treatment ameliorated most of the macroscopic and some of the histopathological indices of colonic damage, and restored the activity of the endogenous antioxidant superoxide dismutase in tissue homogenate but did not affect the signs of inflammation measured by the activity of alkaline phosphatase in serum and tissue homogenate, as well as the expression of NF-kB in the colon. In conclusion, the ameliorative effects of fustin in the experimental TNBS-induced colitis might be the result of its antioxidant properties.

1. Introduction

Inflammatory bowel disease (IBD) is a term for two chronic autoimmune conditions – ulcerative colitis and Crohn's disease. In ulcerative colitis, inflammation of the innermost colon layer is observed, while Crohn's disease may affect any part of gastrointestinal tract and all layers of the intestinal wall. IBD is a lifelong chronic condition with frequent relapses which worsen patient's quality of life [1]. IBD morbidity in Europe and the United States is expected to exceed 7 million people in 2030 [2]. The financial burden of IBD in the USA was estimated between 14.6 and 31.6 billion dollars [[3][4]]. Although IBD etiology remains unclear, recent findings show that genetic and environmental factors, the intestinal flora and the immune system are involved in its pathogenesis [[5],[6]].

Colitis induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS) is a commonly used model of IBD. TNBS is a hapten that couples with high molecular weight proteins and renders them to immunogenic. The result is an excessive Th1-mediated inflammatory immune response [7].

Fustin is a flavonoid found in *Cotinus coggygria* Scop. (Eurasian Smoke tree). It possesses anti-inflammatory properties demonstrated in rat models of carrageenan-induced paw oedema [8] and indomethacin-induced gastric ulceration [9].

The aim of the present study was to investigate the effect of fustin isolated from *Cotinus coggygria* heartwood in a rat model of TNBS-induced colitis.

2. Materials and methods

2.1. Plant Material, Extraction, Isolation, and Purification of Fustin

The *Cotinus coggygria* heartwood was collected at the "Deliblatska Peščara" Natural Reserve in Serbia, in May 2022. Plant material was identified at the University of Belgrade by comparison to Herbarium specimen BEOU 17422 at the Institute of Botany and Botanical Garden in Belgrade, Serbia. The heartwood was air-dried and milled to 1 kg wood powder, further extracted 3 times with 10 L of Dichloromethane/Methanol 1:1 for 24 h at room temperature to give 76 g of crude extract, which was subjected to fractionation by Si gel column chromatography (CC). Isolation was performed using the protocol from our previous studies [10]. The crude extract was chromatographed seven times on a Si gel column (750 × 45 mm), with Dichloromethane/Methanol gradient elution from 97/3 to 60/40. The eluates were monitored by Thin layer chromatography (TLC) on aluminium plates precoated with Merck silica gel 60 F254 (0.25 mm thickness), and the fractions rich in fustin were combined. Pure fustin were isolated from fractions obtained at ratio approximately 80/20 by additional semi-preparative Reversed-Phase High-performance liquid chromatography (RP-HPLC) on a Agilent Technologies 1100 Series HPLC-DAD with Zorbax Eclipse XDB-C18 column (80Å, 150 × 9.4 mm, i.d. 5 μm) at 254 nm for detection, and water/acetonitrile system: 0-20 min, 20-37% Acetonitrile; 20-21 min, 37-50% Acetonitrile; 21-27 min, 50% Acetonitrile; and 27-30 min, 50-100% Acetonitrile. Fustin was purified up to 98% on RP-HPLC using the following program: 0-20 min, 25-40% Acetonitrile (Rt= 5.1 min). Fustin purity was proved by Nuclear magnetic resonance (NMR)-Spectroscopy. The NMR spectra were obtained on a Bruker Avance III 500 (500 MHz for ^{1}H; 125 MHz for ^{13}C), in Deuterated methanol as solvent. Chemical shifts (d) were expressed in ppm and coupling constants (J) in hertz (Hz).

2.2. Experimental design

2.2.1. Experimental animals

The experiment was performed on thirty male Wistar rats (weight 150-250 g). The animals were housed in plastic cages at a room temperature 21±1° C and on a 12/12 light/dark cycle. They had a free access to food and water. The animals were allocated to three groups (n=10): Control, TNBS and TNBS+F10. Before colitis induction, rats were deprived of food for 24 hours.

This animal study was approved by Bulgarian Food Safety Agency - approval: Protocol № 23/April 15, 2021; Permission № 305/June 28, 2021. Procedures of animal treatment and experiments were conducted in accordance with the national and international laws and policies (EU Directive 2010/63/EU for animal experiments).

2.2.2. Induction of colitis

For induction of colitis, the procedure described by Morris et al. [11] was used. Combination of ketamine/xylazine applied intraperitoneally was used for anesthesia. TNBS at a dose of 10 mg dissolved in 0.25 ml 50% ethanol were administered by a soft cannula in the colon at a depth of 8 cm from the anus. In the control animals, physiological saline was applied intrarectally. To prevent fluid leakage, animals were placed in a head-down position for 10 min.

2.2.3. Oral treatment

Oral treatment by an orogastric cannula was initiated 24 hours after colitis induction and lasted for 8 days. Fustin, 10 mg/kg, prepared as a suspension in distilled water and Tween 80 (at a volume of 10 ml/kg) was administered to group TNBS+F10. Groups Control and TNBS received the vehicle for fustin (distilled water and Tween 80) at a volume of 10 ml/kg.

2.3. Body weight

The body weight of animals was measured initially 24 hours before colitis induction, and daily for 8 consecutive days after the procedure. Changes in the body weight were calculated.

2.4. Tissue preparation and fractionation

On the 10^{th} day of the experiment, 24 hours after the last treatment, the animals were sacrificed under diethyl ether anesthesia. Blood was collected from the sublingual veins for the preparation of serum for biochemical investigations. Laparotomy was performed. The colon was assessed macroscopically using scoring criteria. Samples of colon tissue were frozen for biochemical investigations and for histopathological examination.

2.5. Macroscopic assessment of colitis

Adhesions of the colon to the adjacent organs and signs of obstruction were recorded. The large intestine from the anus to the caecum was surgically removed. The length (cm), the weight (g), the area of necrosis (mm^2) and the thickening of the wall were measured. The following scores were used [12]: *Adhesions:* 0 = no adhesions, 1 = difficult dissection, 2 = visible adhesions, 3 = "wrapped" colon; *Obstruction:* 0 = no obstruction, 1 = need for gentle manual cleaning, 2 = fecal impaction; *Wall thickening:* 0 = similar to uninflamed intestine, 1 = thicker than normal (1-2 mm), 2 = much thicker than normal (>2 mm).

2.6. Histopathological evaluation of colitis

Histological specimens from the colon were prepared at the Histopathology Laboratory, St. Marina University Hospital – Varna. The colonic samples were fixed in 10% buffered formaldehyde, dehydrated in graded ethanol concentration, embedded in paraffin, and cut into 4 μm sections. Then the sections were stained with hematoxylin and eosin. The degree of histological changes was determined by using the grading system shown on the following table.

Table 1. Evaluation of histological changes using a grading system

Parameter	**Score**
Epithelial cells and glands	0 – normal
	1 – focal destruction of superficial cells and/or glands

	2 – zonal destruction of surface cells and/or zonal loss of crypts
	3 – diffuse ulceration involving the submucosa and/or diffuse loss of crypts
Inflammatory infiltrate	0 – absence of infiltrate
	1 – inflammatory cells subepithelially and in the lamina propria
	2 – the inflammatory infiltrate reaches the muscularis mucosa
	3 – severe and diffuse infiltrate reaching the muscularis mucosa and/or involving the muscularis propria
Edema	0 – absent
	1 – focal
	2 – zonal and/or moderately diffuse
	3 – extensive and severe

2.7. Immunohistochemical evaluation of NF-kB expression

Tissue sections from rat colon, 4 μm thick, were placed on silanized slides. NF-κB expression was determined using a rabbit anti NF-kB-p100 polyclonal antibody (E-AB-32222; Elabscience, USA), diluted 1:200, following the protocol for universal highly sensitive visualization system for antibody detection EnVision FLEX.

Immunohistochemical evaluation was determined semi-quantitatively in 50 cells of each probe using the following score: 1 – no cytoplasmic staining, 2 – weak cytoplasmic staining, 3 – moderate cytoplasmic staining, 4 – strong cytoplasmic staining. The average intensity of the immune reaction was verified in the following way: number of cells of each type x corresponding coefficient (1, 2, 3 or 4) x total number of $cells^{-1}$.

2.8 Biochemical assays

Blood samples were centrifuged at 2000 rpm for 10 min and serum was collected. Colon tissue with weight of 1 g was mixed with 10 ml 50 mM Tris/HCL buffer (pH=7.4) and was homogenized for 3-4 min at 3500 rpm. The homogenate was centrifuged at 4°C and 3000 rpm for 15 min and the supernatant was collected. Using colorimetric kits (Biomaxima – Poland), alkaline phosphatase (AP) levels were determined in serum and tissue homogenate. Colon homogenate the activity of superoxide dismutase (SOD) was measured by colorimetric assay using the Superoxide Dismutase Activity Kit (Product No. 19160, Sigma-Aldrich) and multi-mode microplate reader (Synergy 2, BioTek Instruments, USA).

2.9. Statistical analysis

GraphPad Prism 5 statistical software was used to analyze the data. One-way ANOVA, followed by Dunnett's multiple comparisons test was performed. Results were presented as mean ± SEM. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Body weight

The body weight values and changes are presented on figure 1 and figure 2. Groups TNBS and TNBS+F10 showed a significant reduction in the body weight ($p < 0.001$ vs. Control) within 3 days after the induction of colitis (Figure 2A). In the next 6 days, the weight of the rats belonging to TNBS group increased gradually, and on 9^{th} day, their weight gain was comparable to that of the Control group (Figure 2B). For the same period, the weight gain of fustin-treated animals was greater, although non-significantly different from that of the other two groups (Figure 2B).

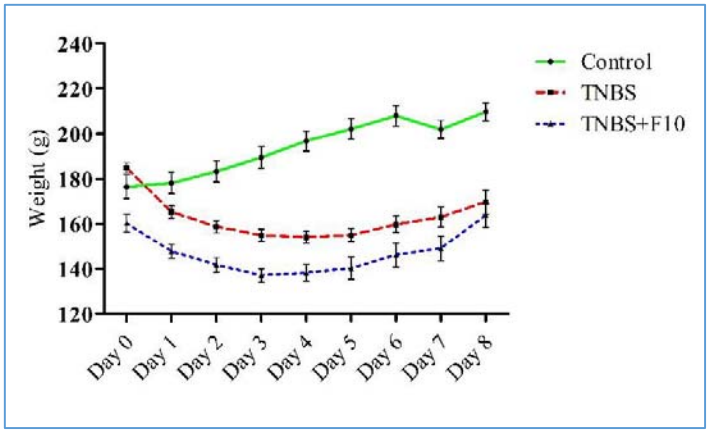

Figure 1. Changes in body weight of animals in a model of 2,4,6-trinitrobenzensulfonic acid (TNBS)-induced colitis in rats and fustin (F) treatment

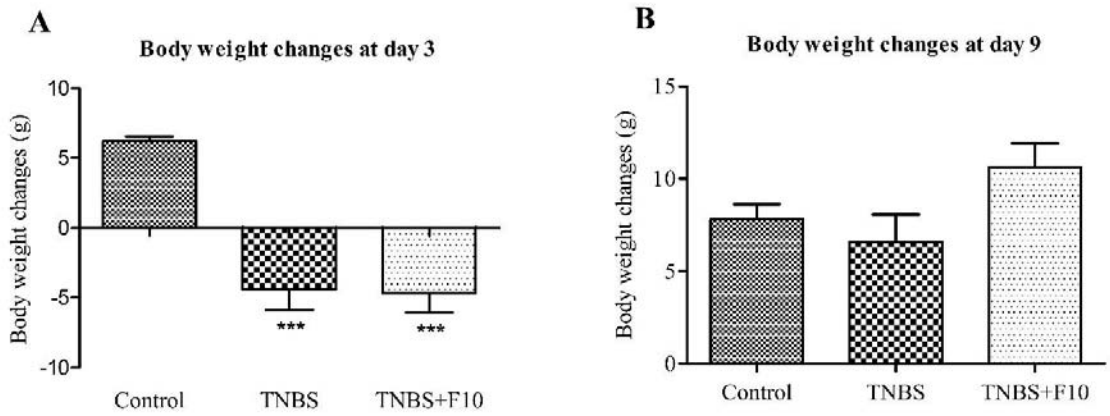

Figure 2. Body weight changes: (A) from day 0 to day 3 and (B) from day 3 to day 9 after 2,4,6-trinitrobenzensulfonic acid (TNBS)-induced colitis in rats and fustin (F) treatment

3.2. Macroscopic assessment of colitis

Pictures presenting the macroscopic appearance of rat colon at the site of TNBS application are shown on figure 3. The colon of animals from group Control was with normal appearance (Figure 3A). In group TNBS (Figure 3B), hemorrhagic ulcerations, covered by fibrinoid necrotic layer were observed. In animals from TNBS+F10 group the ulcerations were reduced (Figure 3C).

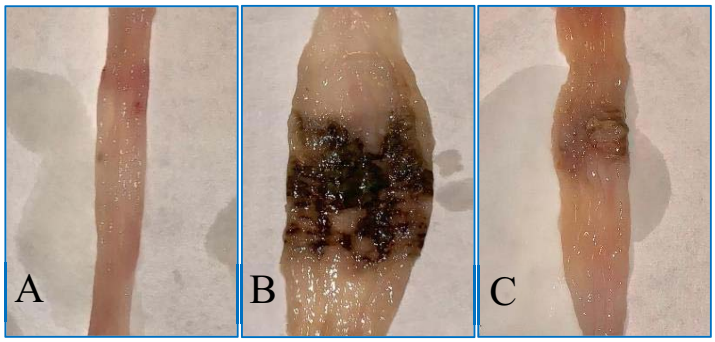

Figure 3. Macroscopic appearance of rat colon in a model of 2,4,6-trinitrobenzensulfonic acid (TNBS)-induced colitis; panel A: Control, panel B: TNBS, panel C: TNBS+F10

The results of the macroscopic assessment are presented on figure 4. In the TNBS group, the adhesions of the colon to adjacent organs were prominent (Figure 4A), the area of necrosis was with a mean dimension of 8.5 ± 0.9 cm^2 (Figure 4B) and the large intestinal wall was thicker than normal (Figure 4F). The weight of the colon was increased ($p<0.001$) (Fig. 4C), the length was decreased ($p<0.001$) (Figure 4D) and the weight/length ratio was significantly higher ($p<0.001$) (Figure 4E) in comparison with group Control.

The treatment with fustin significantly ameliorated the adhesions ($p<0.01$ vs. TNBS) (Figure 4A) and decreased the area of necrosis ($p<0.05$ vs. TNBS) to a mean dimension of 5.6 ± 0.9 cm^2 (Figure 4B). In fustin treated rats, the weight of the colon (Figure 4C) as well as the weight/length ratio (Figure 4E) were significantly decreased ($p<0.05$), compared to TNBS group. Fustin did not significantly change the length of colon (Figure 4D) and the TNBS-induced thickening of the wall (Figure 4F).

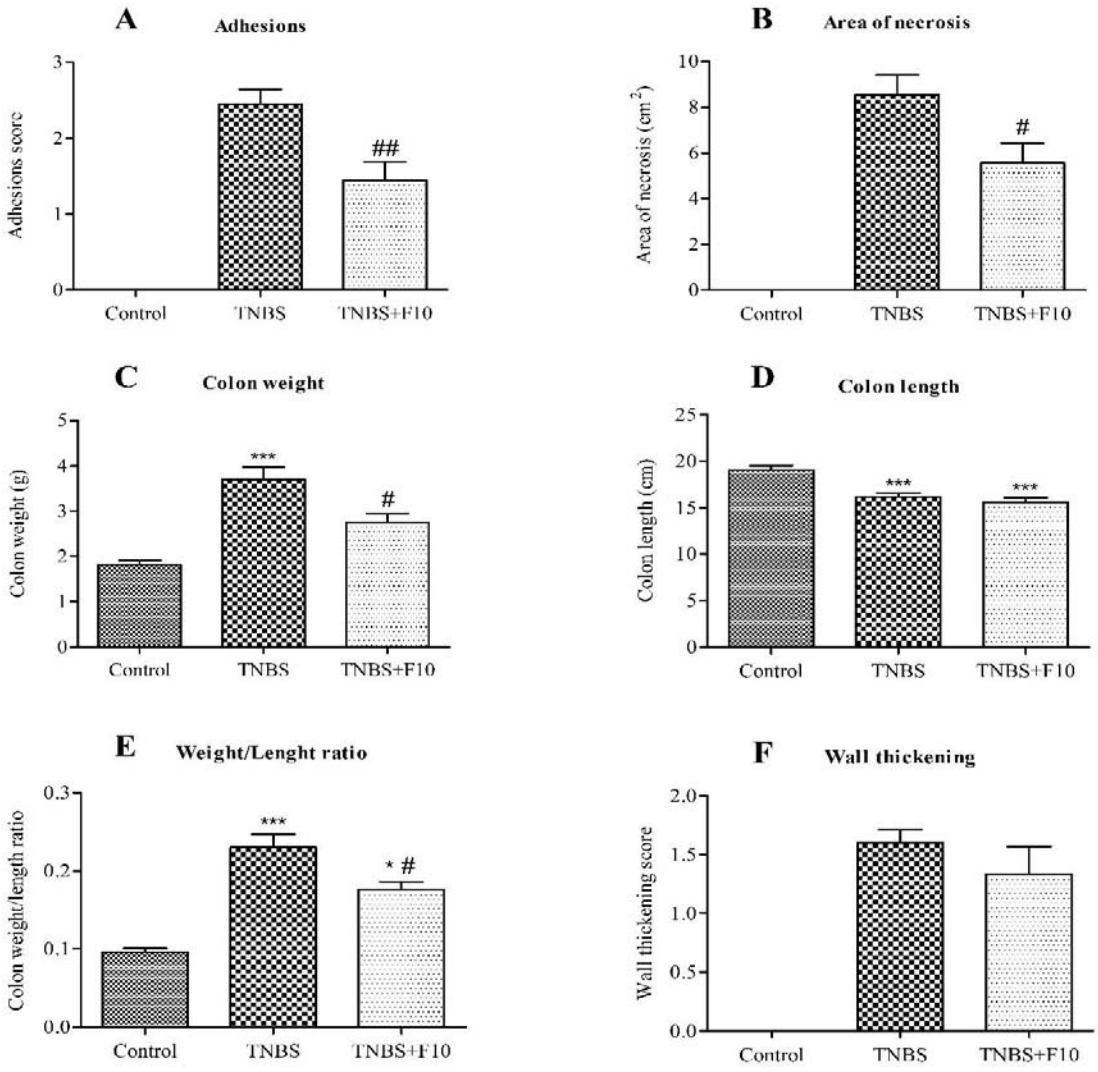

Figure 4. Assessment of the macroscopic signs of 2,4,6-trinitrobenzensulfonic acid (TNBS)-induced colitis in rats and fustin (F) treatment; *$p < 0.05$ vs. Control, ***$p < 0.001$ vs. Control, $^{\#}p < 0.05$ vs. TNBS, $^{\#\#}p < 0.01$ vs. TNBS

3.3. Histopathological evaluation and microscopic scoring of colitis

The colonic mucosa of the animals from the control group had a preserved histological structure (Figure 5A). In TNBS group, diffuse areas of ulceration, marked and diffuse inflammatory infiltrate in all layers of the wall, as well as focal edema were observed (Figure 5B). The treatment with fustin was able to reduce the mucosal destruction and inflammatory infiltration in two of the animals (22%) (Figure 5C).

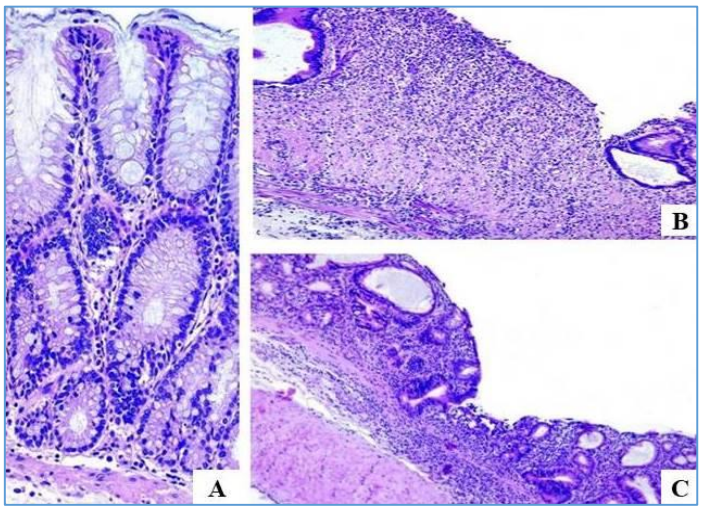

Figure 5. Microscopic appearance of colon in a rat 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model; A. Control – Normal intestinal wall, B. TNBS – Diffuse destruction of intestinal wall, C. TNBS + Fustin 10 mg/kg – Zonal crypt loss; H&E staining; magnification: A x 320, B and C x 200

In the control group, there were no signs of epithelium destruction, inflammatory cells infiltration and edema. Microscopic assessment showed the highest score of mucosal damage, inflammatory cell infiltrate and edema in TNBS group (Figure 6). Fustin at a dose of 10 mg/kg significantly reduced the edema severity ($p<0.05$ vs. TNBS group) (Figure 6C).

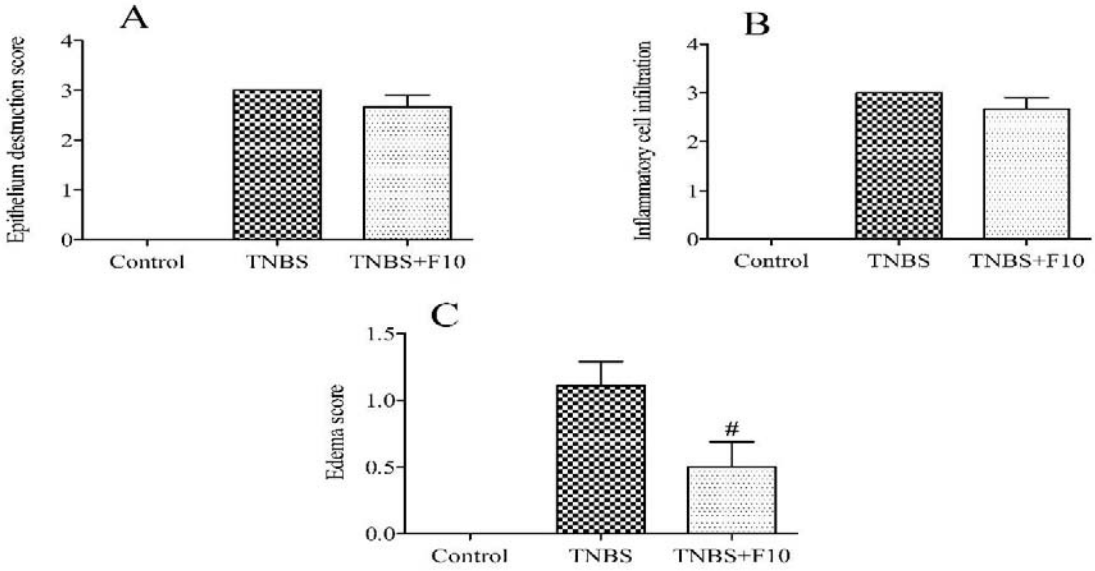

Figure 6. Scoring of histopathological signs of damage in a rat 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model and fustin (F) treatment; $^{\#}p < 0.05$ vs. TNBS

3.4. Immunohistochemical evaluation of NF-kB expression

An increase in the cytoplasmic expression of NF-κB was observed in TNBS group (Figure 7B and 7D, Figure 8), compared to the control group ($p < 0.001$) (Figure 7A). The expression of NF-κB in the mucosa of TNBS+F10 group was reduced (Figure 7C and E) but the difference was not statistically significant (Figure 8). At the edges of ulcers in both TNBS and TNBS+F10 groups, NFκB expression remained high (Figure 7D and 7E).

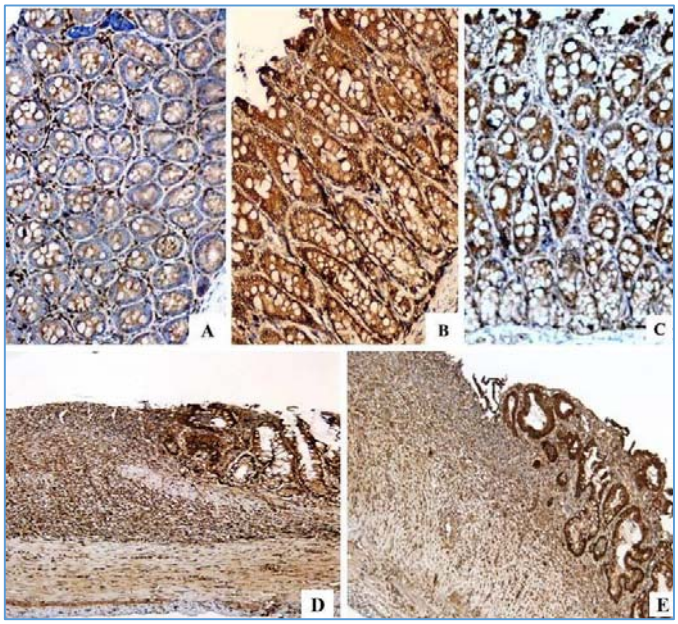

Figure 7. Immunohistochemical staining for NF-κB expression in a rat model of 2,4,6-trinitrobenzensulfonic acid (TNBS)-induced colitis and fustin (F) treatment; A – Control group, B and D – TNBS group, C and E – TNBS+F10 group; magnification x 200

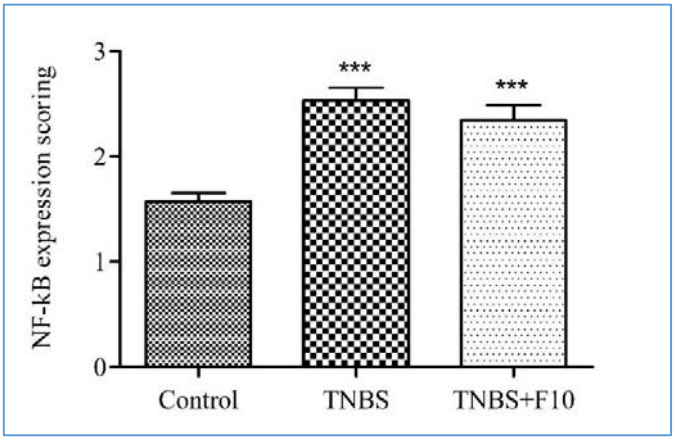

Figure 8. NF-κB expression scoring in a rat model of 2,4,6-trinitrobenzensulfonic acid (TNBS)-induced colitis and fustin (F) treatment; ***$p < 0.001$ vs. Control

3.5. Biochemical measurement

3.5.1 Alkaline phosphatase (AP)

There was no significant change of the serum AP activities in TNBS and TNBS+F10 groups compared to

the control value. AP activity in tissue homogenate was increased by 594.7 % ($p < 0.001$) and 655% ($p < 0.001$) in TNBS and TNBS+F10 groups, respectively, in comparison with the Control group (Figure 9).

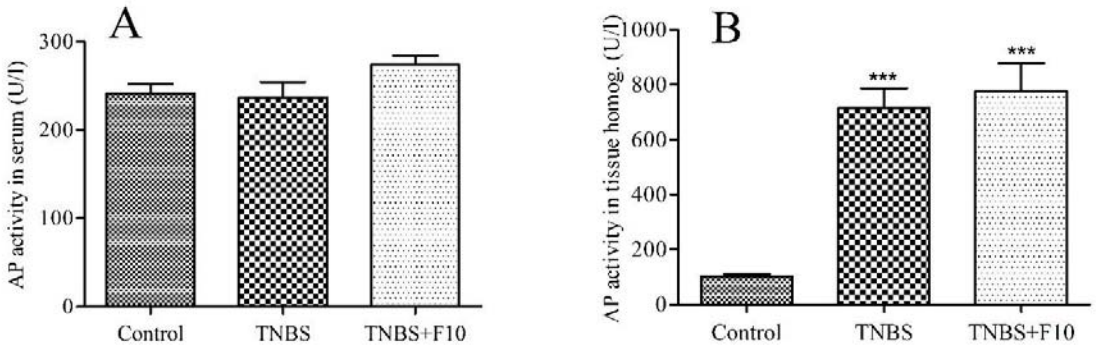

Figure 9. Alkaline phosphatase activity (U/I) in serum (panel A) and colon tissue homogenate (panel B) in a rat model of 2,4,6-trinitrobenzensulfonic acid (TNBS)-induced colitis and fustin (F) treatment; ***$p < 0.001$ vs. Control

3.5.2. Superoxide dismutase (SOD)

TNBS group showed a decreased level of SOD ($p < 0.01$) in tissue homogenate compared to the Control group. In fustin-treated rats, the activity of SOD was increased in comparison with TNBS group and did not differ significantly from the control value (Figure 10).

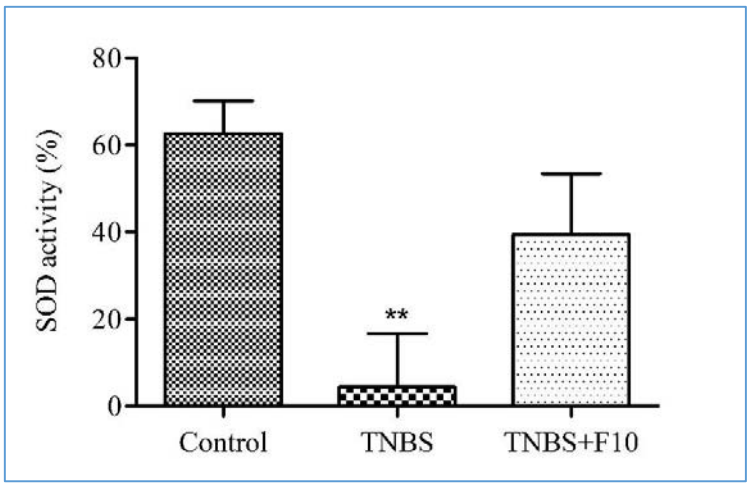

Figure 10. Superoxide dismutase (SOD) activity in colon tissue homogenate in a rat model of 2,4,6-trinitrobenzensulfonic acid (TNBS)-induced colitis and fustin (F) treatment; **$p < 0.01$ vs. Control

4. Discussion

TNBS-induced colitis is a widely used model to mimic the morphological changes in IBD. This experimental model allows investigation of the pathogenesis and treatment options in human ulcerative colitis and Crohn's disease. Ethanol disrupts the mucosal barrier and facilitates TNBS penetration [13] and interaction with tissue proteins stimulating Th1-mediated immune reaction.

The experimental data regarding the key role of oxidative stress in IBD pathogenesis are constantly increasing. Reactive oxygen species are involved mainly in the inflammation and consecutive tissue damage [[14],[15],[16]]. Inflammation is initiated by inflammatory cells migration in the intestinal mucosa leading

to stimulation of free oxygen [[17],[18]] and nitrogen species formation [[19],[20]]. These reactive species cause tissue damage leading to attraction and accumulation of more inflammatory cells. The superoxide ion is the trigger factor in the free radical cascade. It is converted to hydrogen peroxide, hypochloric acid and water. The enzyme SOD converts superoxide anion to hydrogen peroxide and represents a crucial part of the endogenous antioxidant system. In patients with IBD, decreased production of Cu/Zn-SOD was observed clinically [[21],[22]]. In this experiment, a significant reduction of SOD levels was present in TNBS-treated animal group.

Some free oxygen species trigger activation of transcription factors like NF-κB. NF-κB represents a key player in immune-mediated inflammatory reaction including the production of cytokines and immunoglobulins. NF-κB production is stimulated by TNF-alpha, free radicals, bacterial lypopolysaccharides etc.

Colon inflammation is characterized also by stimulation of AP enzymatic activity [23]. AP is considered a biochemical marker of intestinal inflammation. The application of anti-inflammatory drugs like prednisolone and sulphasalazine was able to reduce AP levels in TNBS-induced model of colitis in rats [23]. Intestinal microflora damage is another important factor in IBD pathogenesis. Suppression of protective probiotic bacteria leads to stimulation of pathogenic species growth and impairment of the immune tolerance [24]. An attempt to correct the intestinal microflora balance represents an important part of IBD treatment.

Fustin, applied at a dose of 10 mg/kg, demonstrated a reduction of macroscopic indices of colonic damage. In the fustin-treated group the area of necrosis was reduced, as well as the adhesions to adjacent tissues and the intestinal wall thickness. Microscopic indices for colon damage like epithelial destruction and inflammatory cells infiltration were slightly reduced, and edema was significantly reduced in fustin-treated group. An anti-inflammatory activity of fustin has been observed by other authors in a model of adjuvant-induced arthritis [25]. In the above-mentioned research, the anti-inflammatory effect was linked to reduced levels of pro-inflammatory cytokines like TNF-alpha and Il-6, inhibition of oxidative stress (measured by malondialdehyde, catalase, gluthatione, SOD), reduced prostaglandin E2, myeloperoxidase and nuclear factor-erythroid-2–related factor 2 action.

The data from the present experiment allow several explanations for the beneficial effects of fustin. Fustin treatment caused an elevation of the levels of the antioxidant enzyme SOD. Similar results were observed after the application of the stilbenoid resveratrol in IBD patients [26] and also of the flavonone naringenin in an animal model of acetic-acid induced colitis [27]. SOD treatment was also found to be beneficial in experimental colitis models [[28],[29]].

There is an abundant evidence from animal models of IBD of the inhibitory activity of flavonoids on NF-kB signaling pathway [[30],[31],[32]]. However, in this experiment fustin did not cause a significant reduction of NF-kB expression.

In this experiment, fustin had also no effect on AP activity which is a marker of intestinal inflammation.

Some flavonoids exert a beneficial effect on gut microbiota. Hesperetin was able to improve the inflammatory symptoms in a murine model of ulcerative colitis by regulation of intestinal microbiota balance [33]. Similar results were observed with epigallocatehin gallate in a murine model of DSS-induced colitis [34]. More investigation is needed to assess the possibility such a mechanism to be involved in the effect of fustin in TNBS-induced rat colitis model.

5. Conclusion

Fustin treatment at a dose of 10 mg/kg reduced the severity of 2,4,6-trinitrobenzensulfonic acid-induced colitis in rats. This effect was manifested by the decrease of the necrotic lesions and mucosal edema. However, the inflammatory markers, such as inflammatory cells infiltration, NF-kB expression and the intestinal activity of alkaline phosphatase, remained unchanged. At the same time, the activity of the endogenous antioxidant enzyme superoxide dismutase was elevated in the colon tissue which might lead to

the conclusion that the observed ameliorative effect of fustin in this study could be the result of its antioxidant properties.

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