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Original Contribution

THE BIOFLAVONOID FUSTIN ISOLATED FROM *COTINUS COGGYGRIA* HEARTWOOD ALLEVIATES TRINITROBENZENESULFONIC ACID-INDUCED COLITIS IN RATS BY SUPPRESSION OF NF-κB SIGNALLING PATHWAYS

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ABSTRACT

PURPOSE: To assess the impact of 20 mg/kg orally administered fustin on colon histology and immunohistochemical expression of nuclear factor kappa B (NF-κB) in 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis model in rats. METHODS: Cotinus coggygria heartwood was used to obtain fustin and then the substance was purified. There were three groups of Wistar rats – Control, TNBS and TNBS+F20. To induce colitis, 10 mg TNBS was applied rectally. Control rats were applied saline rectally. Fustin (20 mg/kg) was administered daily orally for 8 days to animals from TNBS+F20 group while the other groups received the vehicle for fustin. At the end of the experiment, colon samples were collected and the histological changes in the colon were investigated under light microscopy after staining with hematoxylin-eosin. Polyclonal antibodies were utilized to determine the expression of NF-κB immunohistochemically. RESULTS: In the TNBS group, histological analysis revealed widespread epithelial destruction, inflammatory infiltration throughout all layers of the colon wall, and localized oedema, along with a significant increase in cytoplasmic NF-κB expression compared to the Control group ($p<0.001$ vs. Control). Fustin treatment significantly lowered the microscopic scores for epithelial destruction ($p<0.001$ vs. TNBS), colonic inflammatory infiltration ($p<0.01$ vs. TNBS), and oedema ($p<0.01$ vs. TNBS). Additionally, immunohistochemical expression of NF-κB was notably reduced in the fustin-treated group ($p<0.001$ vs. TNBS). CONCLUSIONS: The Cotinus coggygria-derived flavonoid fustin, orally administered at a dose of 20 mg/kg, was effective in mitigating the microscopic and immunohistochemical signs of TNBS-induced colitis.

Key words: flavonoid, fustin, Cotinus coggygria, TNBS, colitis

INTRODUCTION

Ulcerative colitis and Crohn's disease are two distinct clinical disorders unified under the condition "inflammatory bowel disease (IBD)".

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The incidence of IBD has been rising worldwide, with local geographic and ethnic differences identified (1). Since IBD is linked to disabilities, researchers are constantly testing novel treatment possibilities *in vitro* and *in vivo* on animals.

A common experimental model of colitis in animals involves rectal administration of trinitrobenzenesulfonic acid (TNBS). TNBS-induced colitis is characterized by an immune-

mediated pathophysiology. Therefore, it could be used to study the profile of cytokines secreted and to elucidate the mode of action of new therapeutic agents (2).

The various health benefits of polyphenols, which are among main constituents of colorful parts of plants, have attracted significant scientific attention. They are reported to exert numerous beneficial effects, among which anti-inflammatory and antioxidant. Additionally, these phytochemicals are quite safe. They are undergoing extensive preclinical and clinical testing. Fustin is a polyphenol, classified as dihydroflavanol. It is abundant in *Cotinus coggygria* heartwood and in the plant *Toxicodendron vernicifluum*. Studies have reported that fustin exerts antihyperglycemic, antidyslipidemic and antioxidant effects as well as protective effects on the central nervous system and the gastrointestinal tract (3, 4).

The aim of this investigation was to assess the impact of 20 mg/kg orally administered fustin on colon histology and immunohistochemical expression of nuclear factor kappa B (NF-κB) in 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis model in rats.

MATERIALS AND METHODS

Plant material, extraction, isolation and purification of fustin

All procedures for plant material collection, as well as extraction, isolation and purification of fustin (3,7,3',4'-tetrahydroxyflavanone), have been described in detail previously by Gospodinova et al. (5). In general, dry *Cotinus coggygria* heartwood powder was extracted to crude extract, further fractionated by column chromatography screened by analytical thin layer chromatography (TLC). Fustin-rich fractions, detected by nuclear magnetic resonance (NMR) spectroscopy, were used for further purification and isolation of fustin by reverse-phase semi-preparative high-performance liquid chromatography (RP-HPLC).

Experimental animals

Thirty male Wistar rats weighing 200 ± 50 g were used in this experiment. Rodents were housed in standard conditions (ambient temperature, 12/12 hours light/dark cycle) in the Animal house of Medical University – Varna and received food and water ad libitum, except for 24 hours before colitis induction when they were food-deprived. Rats were randomized in three experimental groups each consisting of 10 animals: Control, TNBS and TNBS+F20.

The experiment was permitted by Bulgarian Food Safety Agency with a Protocol № 23 (15.04.2021) and a Permission № 305 (28.06.2021). Animal treatment and experiments followed strictly the national and international laws and policies (EU Directive for animal experiments 63/2010).

Colitis induction

To induce colitis, we followed the procedure of Morris et al. (6). Before the procedure, animals were anesthetized by intraperitoneal injection of ketamine (30 mg/kg) and xylazine (30 mg/kg). Colitis was induced by rectal application of 10 mg 2,4,6-trinitrobenzenesulfonic acid (TNBS) dissolved in 0.25 ml 50% ethanol using a soft cannula inserted at 8 cm from the anus. Control rats received saline using the same method. After this manipulation, rats were placed in an upside-down position for 10 min in order to prevent fluid leakage.

Treatment

The animal treatment was performed by orogastric tube daily in the course of 8 days, starting 24 h after the induction of colitis. Group TNBS+F20 was applied fustin at a dose of 20 mg/kg suspended with Tween 80 in distilled water (10 ml/kg). The other groups (Control and TNBS) were treated with 10 ml/kg of the vehicle for fustin (distilled water and Tween 80).

Preparation of tissue samples

Animals were sacrificed under diethyl ether anesthesia 24 h after the last treatment, 10 days after the onset of the experiment. Colon samples were obtained after laparotomy and preserved for further histopathological and immunohistochemical investigations.

Histopathological evaluation

Colon samples were used for preparation of histological specimens at the Histopathology Laboratory, St. Marina University Hospital – Varna. For fixation of the specimens, 10% buffered formaldehyde was used, after which graded ethanol concentration was applied to dehydrate them. Colon samples were embedded in paraffin, and cut into 4 μm thick sections. Hematoxylin and eosin were used for staining. Microscopic changes were assessed according to the following indices: epithelium destruction, colonic inflammatory infiltration and oedema. The mean scores of these indices were calculated for each group.

The following grading system was used to evaluate the epithelium destruction (appearance of 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.

The grading for the infiltration with inflammatory cells was as follows: 0 – absence of infiltrate; 1 – inflammatory cells found subepithelially and in the lamina propria; 2 – the inflammatory infiltrate reaching the muscularis mucosae; 3 – severe and diffuse infiltrate reaching the muscularis mucosae and/or involving the muscularis propria.
The presence of oedema was graded as: 0 – absent; 1 – focal; 2 – zonal and/or moderately diffuse; 3 – extensive and severe.

NF-κB expression, assessed immunohistochemically

Colon sections, 4 μm thick, were placed on silanized slides. To determine the expression of NF-κB, a rabbit anti-NF-κB-p100 polyclonal antibody E-AB-32222 purchased from Elabscience, USA was used. Antibodies were diluted 1:200 according to the protocol for EnVision FLEX universal highly sensitive visualization system for antibody detection.

According to a semi-quantitative method, to assess the immunohistochemical cytoplasmic staining, 50 cells of each probe were used with the respective scoring of the staining: 1 – absent, 2 – weak, 3 – moderate, 4 – strong. To determine the average intensity of the immune reaction, the following formula was applied: number of cells of each type x corresponding coefficient (1, 2, 3 or 4) x total number of cells^{-1}.

Statistical analysis

For data analysis, GraphPad Prism 5 statistical software was used. The statistical analysis used was one-way ANOVA, followed by Dunnett's multiple comparisons post-test. Representation of results is as mean ± SEM. Statistical significance is accepted at a value of $p<0.05$.

RESULTS

Histopathological assessment and microscopic scoring of colitis

The animals in the Control group showed a normal histological structure of the colonic mucosa (**Figure 1A**). In the TNBS group, diffuse ulcerations were observed, accompanied by extensive inflammatory infiltration throughout all layers of the colon wall and localized oedema (**Figure 1B**). Fustin treatment led to a reduction of epithelial damage and inflammatory infiltration (**Figure 1C**).

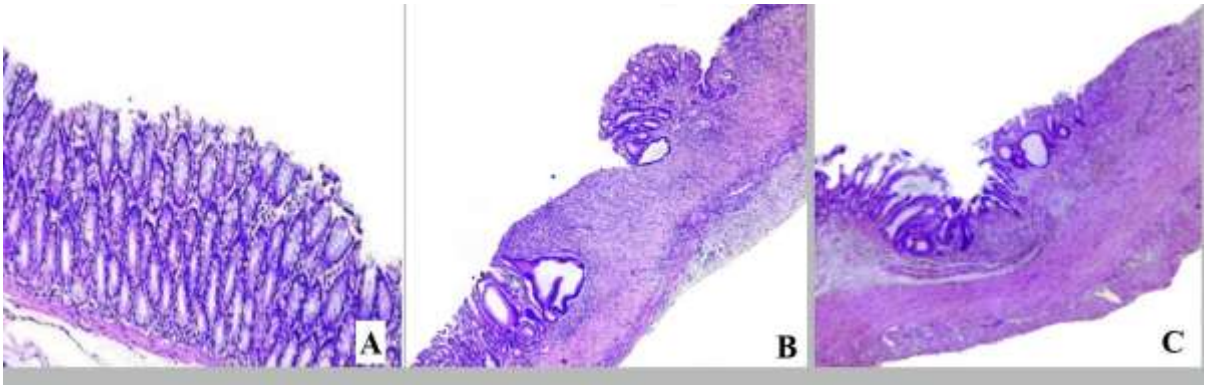

Figure 1. The microscopic images of the rat colon in a colitis model induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS): A. Control – Normal structure of the intestinal wall, B. TNBS – Widespread damage to the intestinal wall, C. TNBS + Fustin 20 mg/kg – Zone specific loss of crypts. The pictures were obtained using H&E staining at 200x magnification

In the Control group, there were no signs of epithelial damage, inflammatory cell infiltration, or oedema. Microscopic analysis showed that the TNBS group had the highest levels of mucosal damage, inflammatory cell infiltration, and oedema. When compared to the TNBS group, treatment with fustin at a dose of 20 mg/kg significantly reduced epithelial and glandular destruction ($p<0.01$), inflammatory cell infiltration ($p<0.01$), and the severity of oedema ($p<0.001$) compared to the TNBS group. The results are shown in **Figure 2**.

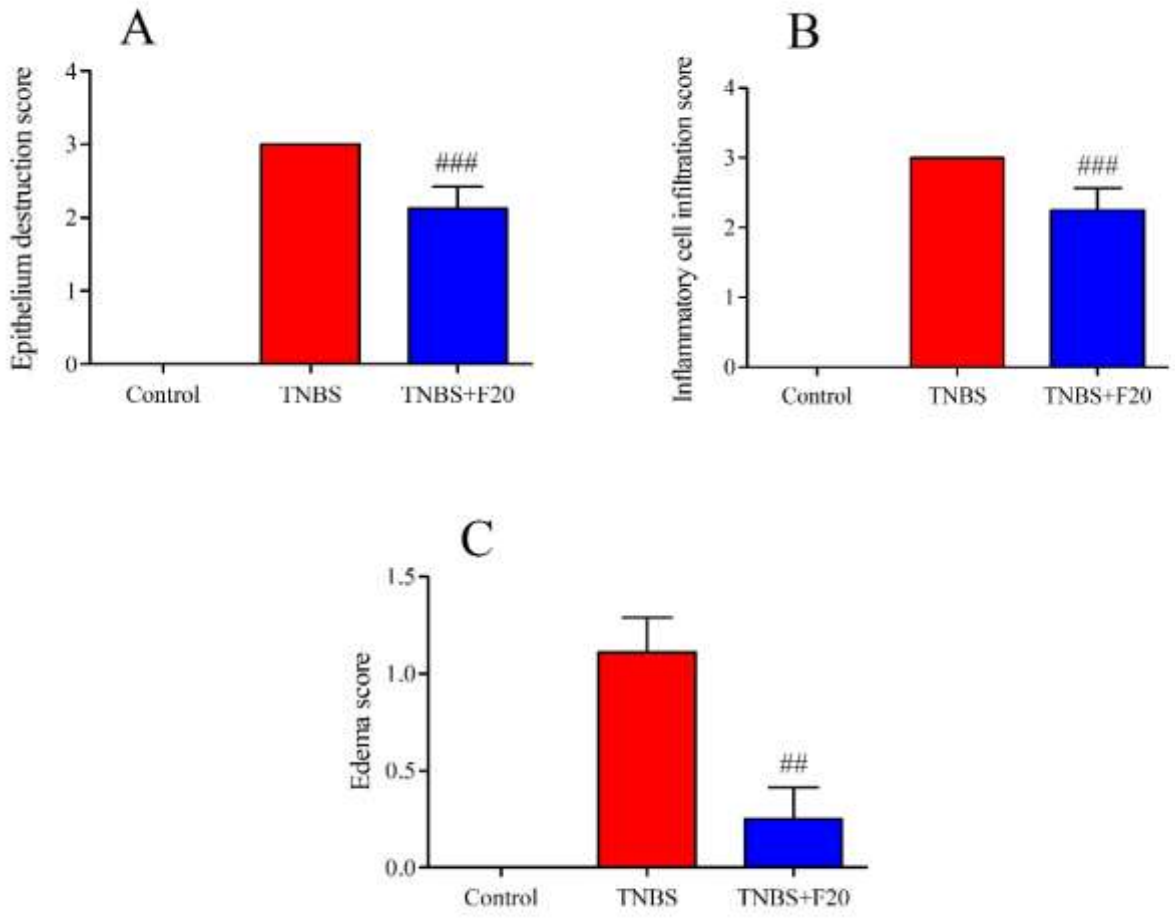

Figure 2. Assessment of histopathological markers of injury in a rat model of colitis induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS) and fustin (F) treatment. ##p < 0.01 compared to TNBS; ###p < 0.001 compared to TNBS

Assessment of NF-κB expression

In the TNBS group, cytoplasmic NF-κB expression was significantly elevated (**Figure 3B** and **Figure 4**) ($p < 0.001$) compared to the Control group (**Figure 3A**). The NF-κB expression in the TNBS+F20 group (**Figure 3C** and **Figure 4**) was markedly reduced ($p < 0.001$ vs. TNBS) approaching levels seen in the Control group.

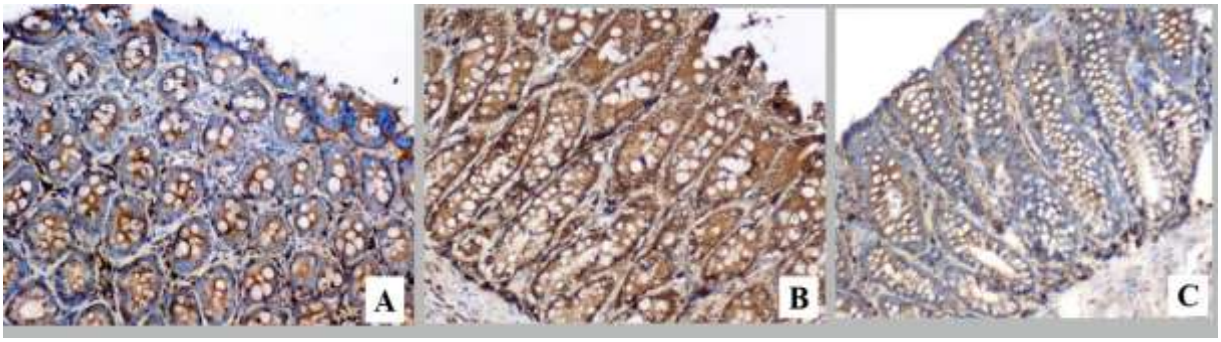

Figure 3. NF-κB expression evaluated through immunohistochemical staining in a rat colitis model induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS) and fustin (F) treatment: A – Control group, B – TNBS group, C – TNBS+F20 group; magnification 200x

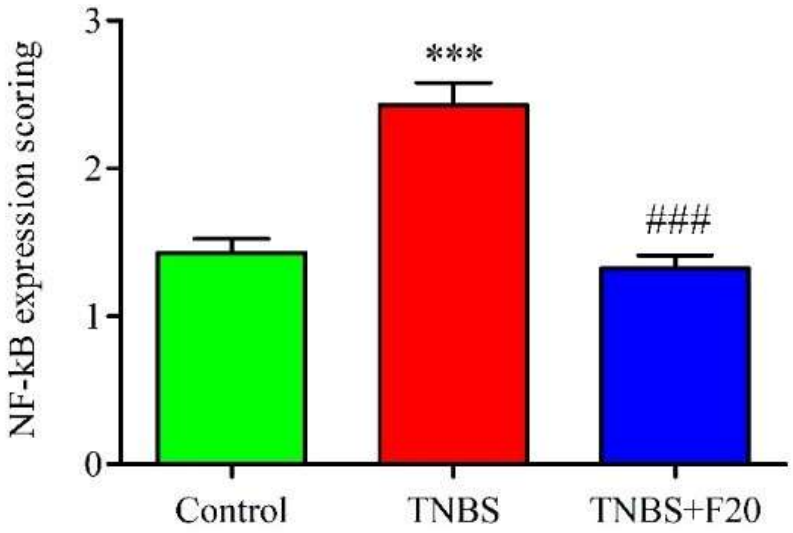

Figure 4. The NF-κB expression score in a rat colitis model induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS) and treated with fustin (F). ***p < 0.001 compared to the Control group; ###p < 0.001 compared to the TNBS group.

DISCUSSION

TNBS-induced colitis is an experimental model that reproduces the histological and immunohistochemical signs as well as the clinical symptoms of IBD. In this model, ethanol administration is implemented in order to potentiate the penetration of TNBS through the intestinal mucosal layer and to produce immune response mediated by Th1-lymphocytes (Th1-Ly) (7). Th1-Ly activation causes release of cytokines (TNF-α) and consecutive tissue destruction. TNF-α is a crucial component in the pathogenesis of IBD (8).

The current experiment involved rectal TNBS administration, which resulted in microscopic alterations characteristic of colitis – wall oedema, infiltration of inflammatory cells and epithelial damage. Treatment with 20 mg/kg fustin reversed these alterations and improved the microscopic indicators of intestinal injury. Moreover, fustin-treated rats showed considerably lower expression of NF-κB in the colonic samples.

These results are in line with earlier research on the role of flavonoids in colitis triggered by TNBS. Flavonoids are plant compounds with the potential to be employed as therapeutic agents in IBD because they boost the body's natural antioxidant defence and reduce inflammation. Reactive oxygen species (ROS) have unpaired electrons and are extremely reactive chemicals that can interfere with cell function by interacting with proteins, lipids, and DNA in the cell membrane, thus, they can produce oxidative stress. ROS also trigger inflammatory response and tissue damage (9-11). A vicious cycle is created when inflammatory cells penetrate the mucosa and produce additional free oxygen (12, 13) and nitrogen species (14, 15). This attracts more inflammatory cells, perpetuating the cycle.

The leading mechanism of the anti-inflammatory activity of the flavonoids in TNBS-colitis model is the inhibition of toll-like receptor 4 (TLR4)-mediated activation of NF-κB. TLR4 is overexpressed in IBD and studies have identified its crucial role in the development of experimental colitis. In inflammation, TLR4 activation triggers both innate (mediated by the intestinal epithelial cells, macrophages/ monocytes, neutrophils, dendritic cells) and adaptive (mediated by the T-cells and B-cells) immune response, leading to NF-κB recruitment and production of pro-inflammatory molecules such as IL-1, TNF-α, COX-2 and ICAM-1 (16, 17). Several flavonoids, including morin (18), kaempferol, epigallocatechin gallate, and naringenin (19), have been shown to inhibit NF-κB activation in stomach ulceration and colitis experimental models.

The imbalance of the gut microbiota is another factor implicated in the pathophysiology of IBD (20). By blocking NF-κB signalling pathways and reestablishing the proper balance of the gut microbiota, natural products have been shown to reduce the severity of experimental colitis

(21). In a model of indomethacin-induced gastric ulceration, the amelioration of inflammation by fustin was underlied by reduction of NF-κB expression (4).

CONCLUSION

The *Cotinus coggygria*-derived polyphenol fustin, orally administered at a dose of 20 mg/kg, was effective in mitigating the microscopic and immunohistochemical signs of TNBS-induced colitis. The observed effect was attributed to the inhibition of the NF-κB pro-inflammatory cascade.

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