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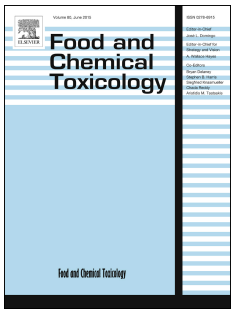

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Aronia melanocarpa Fruit Juice Ameliorates the Symptoms of Inflammatory Bowel Disease in TNBS-induced Colitis in Rats

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Abbreviations: AMFJ, *Aronia melanocarpa* fruit juice; CD, Crohn's disease; IBD, inflammatory bowel disease; IL, interleukin; NF-κB, nuclear factor-kappa B; ROS, reactive oxygen species; S, sulfasalazine; TBARS, thiobarbituric acid reactive substances; TNBS, trinitrobenzensulfonic acid; TNF-α, tumor necrosis factor-α; UC, ulcerative colitis.

1. Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory condition within the gastrointestinal tract with two major forms: ulcerative colitis (UC) and Crohn's disease (CD). Inflammation and ulceration in UC are limited to the colon and rectum, while in CD they are throughout the whole gastrointestinal tract.

The exact provocative etiological factors and pathogenic mechanisms in IBD have not been fully elucidated. There is a dysfunction of the intestinal mucosal barrier resulting in an increased permeability (Amasheh et al., 2009). This process is usually a consequence of the destruction of tight junctions triggered by oxidative stress (Xu et al., 2007), bacterial lipopolysaccharides (Sheth et al., 2007) and inflammatory mediators such as cytokines (Petecchia et al., 2012). As a result, antigenic determinants derived either from food or bacteria can overcome the barrier and provoke an intestinal immune response and tissue damage (Neurath and Schürmann, 2000).

Animal models of IBD are an important tool to study the disease pathogenesis and to test new therapies. 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis is a commonly used model of IBD. Instilled in the colon, TNBS causes severe inflammation and ulceration. TNBS serves as a hapten. When coupled with high molecular weight tissue proteins, it renders them immunogenic and leads to acute Th1 inflammation (Elson et al., 1995) and secretion of various potent pro-inflammatory cytokines (da Silva et al., 2010) such as tumor necrosis factor-α (TNF-α) and interleukins (ILs) (Takagi et al., 2010).

Oxidative stress plays an important role in the pathogenesis of IBD (Girgin et al., 2000). Reactive oxygen species (ROS) are produced and released by immune cells. In this inflammatory condition, the colon is infiltrated by neutrophils which produce superoxide and other strong oxidants such as hypochlorous acid, the result of the catalytic activity of

myeloperoxidase (Winterbourn, 2002). An increase in myeloperoxidase activity can accelerate the progression of IBD (Colón et al., 2001; Cooke et al., 2002).

There are numerous studies reporting that biologically active substances from plants such as polyphenolic compounds have beneficial effects in IBD by alleviating oxidative stress and by controlling the levels of various inflammatory cytokines or mediators including ILs, TNF-α, nitric oxide, nuclear factor-kappa B (NF-κB) and cyclooxygenase-2 (Debnath et al., 2013).

Aronia melanocarpa [Michx.] Elliot (black chokeberry) is a bush originating from North America, nowadays grown also in Eastern Europe. The fruits are extremely rich in phenolic compounds (Oszmianski and Wojdylo, 2005): procyanidins, flavonoids (mainly from the subclass of anthocyanins) and phenolic acids (chlorogenic and neochlorogenic). Well established assays have demonstrated that aronia fruits and fruit extracts act as very powerful antioxidants (Zheng and Wang, 2003; Bermudez-Soto and Tomas-Barberan, 2004; Wu et al., 2004; Oszmianski and Wojdylo, 2005; Valcheva-Kuzmanova et al., 2007; Jakobek et al., 2011; Valcheva-Kuzmanova et al., 2012; Valcheva-Kuzmanova et al., 2014a). *Aronia melanocarpa* fruit juice and aronia berry polyphenols have been shown to exhibit potent anti-inflammatory activities (Borissova et al., 1994; Zapolska-Downar et al., 2011; Martin and Bolling, 2017).

The aim of this study was to investigate the effect of *Aronia melanocarpa* fruit juice (AMFJ) in a rat TNBS-induced colitis model and to compare it with that of sulfasalazine, a standard treatment of IBD.

2. Materials and methods

2.1. Experimental substances

2,4,6-trinitrobenzene sulfonic acid (TNBS), sulfasalazine (S) and all other chemicals were of analytical grade were purchased from Sigma-Aldrich Company (Germany).

AMFJ was produced from *Aronia melanocarpa* Elliot fruits grown in the Balkan Mountains, Bulgaria. Crushing and squeezing of the fruits gave a juice yield of 75%. The juice was filtered, pasteurized at 80 °C for 10 min and stored at 0 °C till the experiment. The contents of phenolic substances in AMFJ were total phenolics, 5461 mg/L as gallic acid equivalents; total proanthocyanidins, 3122.5 mg/L; anthocyanins: cyanidin galactoside, 143.7 mg/L; cyanidin arabinoside, 61.7 mg/L; cyanidin xyloside, 11.6 mg/L and cyanidin glucoside, 4.4 mg/L; phenolic acids: chlorogenic acid, 585 mg/L and neochlorogenic acid, 830 mg/L (Valcheva-Kuzmanova et al., 2014a). Total phenolics were determined spectrophotometrically according to the Folin-Ciocalteu procedure (Singleton and Rossi, 1965), total proanthocyanidins were measured by the method of Howell et al. (2005), the concentrations of individual anthocyanins and phenolic acids were determined by high-performance liquid chromatography methods.

2.2. Animals

The study was carried out on 96 male Wistar rats (weight 300-350 g). During the experiment, the animals were housed in plastic cages in a well ventilated room maintained at 22±1 °C and on a 12/12 light/dark cycle. Rats were deprived of food for 12 h before the induction of colitis but had free access to food (normal pelleted diet) and drinking water throughout the rest of the time. The animals were divided into 6 experimental groups, each of 12 rats: Control, TNBS, TNBS+AMFJ$_{2.5}$, TNBS+AMFJ$_{5}$, TNBS+AMFJ$_{10}$ and TNBS+S.

All procedures concerning animal treatment and experimentation were conducted in compliance with the national laws and policies, in conformity with the international guidelines (EU Directive 2010/63/EU for animal experiments).

2.3. Induction of colitis

Colitis was induced according to the procedure described by Morris et al. (1989). Following a 12 h fast, rats were anesthetized with thiopental (50 mg/kg, dissolved in saline to a volume of 1 mL/kg) administered imtraperitonelly. TNBS (10 mg dissolved in 0.25 ml of 50% ethanol) was inserted in the colon by a soft cannula (external diameter 1.5 mm) at a depth of 8 cm from the anus. Control rats received 0.25 ml of 50% ethanol. The animals were maintained in a head-down position for 10 min to prevent the fluid leakage.

2.4. Oral treatment

The oral treatment of the animals using an orogastric cannula began on the 2nd day (24 hours after the induction of colitis) and lasted till the 14th day of the experiment. The animals from groups Control and TNBS were treated with distilled water (10 mL/kg).

Animals belonging to groups TNBS+AMFJ$_{2.5}$, TNBS+AMFJ$_{5}$, TNBS+AMFJ$_{10}$ were respectively treated with AMFJ at doses of 2.5 mL/kg, 5 mL/kg and 10 mL/kg. The doses of 2.5 mL/kg and 5 mL/kg were diluted with distilled water to a total volume of 10 mL/kg. AMFJ doses were chosen on the basis of our previous experience. These doses had been proven to be protective in different experimental models of organ damage and toxicity in rats and any further increases in the dose had not resulted in a better effect (Valcheva-Kuzmanova et al., 2004; Valcheva-Kuzmanova et al., 2005; Valcheva-Kuzmanova et al., 2012; Valcheva-Kuzmanova et al., 2014b). Taking into account the water content of fruits, the juice doses of 2.5 mL/kg, 5 mL/kg and 10 mL/kg corresponded to dry residue base of 0.67 g/kg, 1.34 g/kg and 2.68 g/kg, respectively.

Animals from group TNBS+S received sulfasalazine at a dose of 400 mg/kg dissolved in distilled water to a volume of 10 mL/kg.

2.5. Assessment of colitis

2.5.1. Body weight

Animal body weights were recorded at the beginning and at the end of the experiment. Weight gain or reduction were calculated.

2.5.2. Tissue preparation and fractionation

On the 15th experimental day, 24 hours after the last treatment, the animals were anesthetized with diethyl ether. Blood was collected from the sublingual veins for the preparation of serum for biochemical investigations.

After the dacapitation of the anesthetized animals, laparotomy was performed. The colon was removed, cleansed of the adjacent tissues and its length was measured. Then, the colon was cut longitudinally and washed in saline. The weight of the colon was measured and the severity of colitis was evaluated using macroscopic scoring criteria (Daddaoua et al., 2005).

Samples of colon tissue from the site of injury (or a corresponding site if no detectable injury) were frozen for biochemical investigations.

In a similar way, samples of colon tissue were taken for histopathological examination. They were fixed in 10% neutral-buffered formaldehyde solution.

2.5.3. Macroscopic assessment of colitis

The severity of colitis was assessed by the following parameters: colon length (cm), colon weight (g), colon weight/length ratio and area of necrosis (cm^2).

Apart from these, scoring criteria were used for the macroscopic evaluation of adhesions and wall thickening (Daddaoua et al., 2005) (Table 1).

Table 1. Macroscopic score of colonic injury

Parameter	Score
Adhesions	0 = No adhesions

	1 = Difficult dissection 2 = Visible adhesions 3 = "Wrapped" intestine
Thickening	0 = Similar to uninflamed intestine 1 = Thicker than normal (~1–2 mm) 2 = Much thicker than normal (>2 mm)

2.5.4. Microscopic assessment of colitis

Fixed tissues were embedded in paraffin, cut into sections and placed on microscope slides. Slides were stained with hematoxylin and eosin (H&E) for histopathological investigation which was performed by light microscopy.

Scoring criteria were used for evaluation of the destruction of epithelial surface and glands, as well as for the inflammatory cell infiltration (Elli et al., 2011) (Table 2).

Table 2. Microscopic score of colonic injury

Parameter	Score
Epithelium and glands	0 = Normal 1 = Focal destruction of epithelial surface and/or glands 2 = Zonal destruction of epithelial surface and/or zonal crypt loss 3 = Diffuse mucosal ulceration involving submucosa and/or diffuse crypt loss
Inflammatory cell infiltration	0 = Absence of infiltrate

	1 = Subepithelial infiltrate and infiltrate in the lamina propria 2 = Infiltrate reaching the muscolaris mucosae 3 = Severe and diffuse infiltrate reaching the submucosa and/or involving the muscolaris propria

2.5.5. Biochemical investigations

Serum was produced by centrifugation of blood at 2000 rpm for 10 min. Samples of colon tissue were homogenized with ice cold Tris/HCl, 50 mM, pH 7.4 (1:10). The homogenates were centrifuged (2000 rpm, 10 min, 4 °C) and the supernatants were used.

Thiobarbituric acid reactive substances (TBARS) in rat serum and colonic tissue homogenates were determined spectrophotometrically after the method of Ohkawa et al. (1979) using Aurius 2021 UV-VIS spectrophotometer, Cecil Instruments Ltd, UK. The method measures the color produced by the reaction of thiobarbituric acid with lipid peroxides (TBARS) at 532 nm. TBARS concentration was determined in nmol/mL serum and nmol/g tissue. Malondialdehyde, the major lipid peroxide obtained in the process of peroxidation of membrane polyunsaturated fatty acids, was used as a standard.

2.6. Statistical analysis

Results are presented as mean ± S.E.M. The data were tested by one-way ANOVA, followed by Dunnett's multiple comparison post test. A level of $p<0.05$ was considered significant. All analyses were performed using GraphPad Prism statistical software.

3. Results

3.1. Body weight

On the 15th experimental day, control animals showed an increase in body weight while animals belonging to all other groups reduced their weights. The weight loss was significant in TNBS group ($p<0.05$ vs. Control) and was not statistically significant in the groups treated either with AMFJ, or sulfasalazine (Fig. 1).

3.2. Macroscopic colitis evaluation

The macroscopic appearance of the colons of rats belonging to groups Control, TNBS, TNBS+AMFJ$_{10}$ and TNBS+S is illustrated in Fig. 2.

Colons of control rats showed a normal macroscopic appearance (Fig. 2A).

The macroscopic inspection of the colons of TNBS rats showed the presence of edema and hemorrhagic ulcerations (Fig. 2B) with a mean area of necrosis 1.9 ± 0.9 cm^2 (Fig. 3D).

In TNBS group, there was a significant decrease of the colon length ($p<0.05$) (Fig. 3A), a marked increase in the colonic weight/length ratio ($p<0.05$) (Fig. 3C) and a significant increase of the wall thickening score ($p<0.05$) (Fig. 3F) in comparison with the Control group. In rats from TNBS group, adherence of colon to adjacent organs was observed resulting in an adhesions score of 1.1 ± 0.4 (Fig. 3E).

Treatment of animals with AMFJ attenuated the colon shortening (Fig. 3A), the colon weight increase (Fig. 3B) and the elevation of colonic weight/length ratio (Fig. 3C). AMFJ decreased the lesion extension (Fig. 2C, Fig. 3D), reduced the adhesions score (Fig. 3E) and the wall thickening score (Fig. 3F) to such an extent that these indices in the three AMFJ-treated groups were not significantly different from those of the controls. An exception of this was the wall thickening score of TNBS+AMFJ$_{2.5}$ rats which was similar to that of the TNBS rats (Fig. 3F). The effect of AMFJ at doses of 5 mL/kg and 10 mL/kg on mascoscopic signs of colitis was comparable to that of sulfasalazine (Fig. 2D, Fig. 3).

3.3. Histopathological examination and microscopic scoring for colitis evaluation

The histological examination of the colons of control rats showed normal microscopic structure (Fig. 4A).

TNBS caused a diffuse destruction of intestinal wall and inflammatory cell infiltration involving the muscularis propria (Fig. 4B) with a high epithelium and glands destruction score (Fig. 5A) as well as a high inflammatory cell infiltration score (Fig. 5B).

AMFJ dose-dependently attenuated the microscopic signs of colonic damage. The highest was the effect of the 10 mL/kg AMFJ dose. Thus, in TNBS+$AMFJ_{10}$ group, the predominant microscopic results showed a zonal destruction of epithelial surface and inflammatory cell infiltration reaching the muscularis mucosae (Fig. 4C). The epithelium and glands destruction scores of rats belonging to TNBS+$AMFJ_5$ and TNBS+$AMFJ_{10}$ groups were significantly lower ($p<0.01$) than the respective score of TNBS group (Fig. 5A). The inflammatory cell infiltration scores of TNBS+$AMFJ_5$ and TNBS+$AMFJ_{10}$ groups were lower than the respective score for TNBS rats, the effect being statistically significant ($p<0.05$ vs. TNBS) at AMFJ dose of 10 ml/kg (Fig. 5B).

Sulfasalazine treatment attenuated the microscopic signs of colonic damage. In TNBS+S rats, the prevailing microscopic result was a zonal destruction of epithelial surface and inflammatory cell infiltration involving the muscularis propria (Fig. 4D). The epithelium and glands destruction score, as well as the inflammatory cell infiltration score of rats belonging to TNBS+S group were lower but not significantly than the respective scores of TNBS group (Fig. 5A, 5B).

3.4. Thiobarbituric acid reactive substances (TBARS)

The concentrations of TBARS in colonic tissue of rats belonging to group TNBS were significantly higher ($p<0.05$) in comparison with the control level (Fig. 6A). Colonic TBARS

concentration of rats belonging to groups TNBS+AMFJ$_{2.5}$, TNBS+AMFJ$_5$, TNBS+AMFJ$_{10}$ and TNBS+S did not differ significantly from the control level.

The serum concentrations of TBARS were not significantly different in all experimental groups (Fig. 6B).

4. Discussion

The current study was undertaken in order to investigate the effect of AMFJ on the severity of TNBS-induced colitis in rats. The effect of AMFJ was compared with that of sulfasalazine, one of the mainstays for treatment of IBD. Poorly absorbed in the gastrointestinal tract, sulfasalazine molecule is cleaved by reductases produced by colonic bacteria to 5-aminosalicylic acid and sulfapyridine. The mechanism by which sulfasalazine acts in IBD is complex. It induces T lymphocyte apoptosis (Doering et al., 2004), serves as a scavenger of ROS (Linares et al., 2011), suppresses NF-kB transcription pathway (Cavallini et al., 2001).

TNBS-induced colitis was chosen as a model in this study because of its significant similarities with human IBD, particularly CD (Antoniou et al., 2016). TNBS causes acute and chronic colitis in combination with ethanol. Ethanol, as a breaker of mucosal barrier, makes possible the interaction of TNBS with the tissue proteins of the colon (Ikeda et al., 2008).

In the current experiment, TNBS caused severe colitis manifested by a decrease of animal weight, as well as colonic ulceration and inflammation proven by a number of macroscopic and microscopic indices. It also caused oxidative stress in rat colons resulting in an increased lipid peroxidation confirmed by the significant elevation of the TBARS concentration in rat colonic tissue.

Numerous studies have reported the role of oxidative stress in the pathogenesis of IBD in humans and experimental colitis in animal models (Cruz et al., 2001; Pavlick et al., 2002; Seril et al., 2003). During the inflammatory process in the colon, there is a chronic presence

of numerous polymorphonuclear neutrophils which produce ROS during phagocytosis causing oxidative burst (Lih-Brody et al., 1996).

In this experiment, the treatment of rats with AMFJ and sulfasalazine ameliorated the severity of the colitis model. Both treatments reduced the animal weight loss. AMFJ dose-dependently decreased the colonic damage proven by macroscopic and microscopic criteria. The effect of AMFJ on most of measured parameters was comparable with that of sulfasalazine while the microscopic scoring showed an even better effect of the 10 mL/kg AMFJ dose in comparison with that of sulfasalazine.

The beneficial effect of the treatments might be at least partly attributed to their antioxidant actions. AMFJ and sulfasalazine prevented the elevation of TBARS in colonic tissue. The antioxidant activity of AMFJ is one of its most extensively studied and well-established activities. AMFJ polyphenols have been reported to serve as radical scavengers (Zheng et al., 2003; Jakobek et al., 2011; Valcheva-Kuzmanova et al., 2012; Valcheva-Kuzmanova et al., 2014a) and to increase the endogenous antioxidants such as reduced glutathione and antioxidant enzymes in different experimental models of organ damage and toxicity (Valcheva-Kuzmanova et al., 2004; Valcheva-Kuzmanova et al., 2005; Valcheva-Kuzmanova et al., 2012). Sulfasalazine has also been shown to act as a ROS scavenger (Linares et al., 2011).

An important pathogenic mechanism of both animal TNBS-induced colitis and human IBD is the T cells-mediated immune response (Elson et al., 1995). T cells and their cytokines contribute to the inflammatory process in these conditions. A recent study has shown that an aronia berry extract significantly inhibited the production of TNF-α by stimulated Jurkat T cells (Martin and Bolling, 2017). On the basis of this investigation, the authors concluded that aronia berry polyphenols could be useful in prevention and treatment of the inflammation in IBD.

The observed ameliorative effects of AMFJ in the present experiment of rat TNBS-induced colitis are in accordance with data for beneficial effects of an aronia berry extract in dextran sodium sulfate-induced ulcerative colitis in mice (Kang et al., 2017). In the cited experiment, aronia extract decreased the production of pro-inflammatory cytokines (IL-6 and TNF-α) (Kang et al., 2017). Similar mechanisms could contribute to the effect of AMFJ in the present experiment.

Moreover, an anti-inflammatory activity of AMFJ was confirmed by the decrease of serum IL-6 in a rat model of amiodarone-induced pulmonary toxicity (Valcheva-Kuzmanov et al., 2014b). The anti-inflammatory activity was the first established pharmacological activity of the natural juice from *Aronia melanocarpa* in a model of rat hind paw inflammation induced by histamine and serotonin (Borissova et al., 1994).

In conclusion, this study demonstrated that the severity TNBS-induced colitis was significantly ameliorated by AMFJ, the effect being comparable to or even higher than that of sulfasalazine. The effect of AMFJ might be attributed to the antioxidant and anti-inflammatory activities of its polyphenolic ingredients.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Figure captions

Fig. 1. Effect of *Aronia melanocarpa* fruit juice (AMFJ) at doses of 2.5, 5 and 10 mL/kg and sulfasalazine (S) on weight gain of rats in a 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model; *p<0.05 vs. Control

Fig. 2. Macroscopic appearance of colons in a rat 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model: A. Control; B. TNBS; C. TNBS+$AMFJ_{10}$; D. TNBS+S

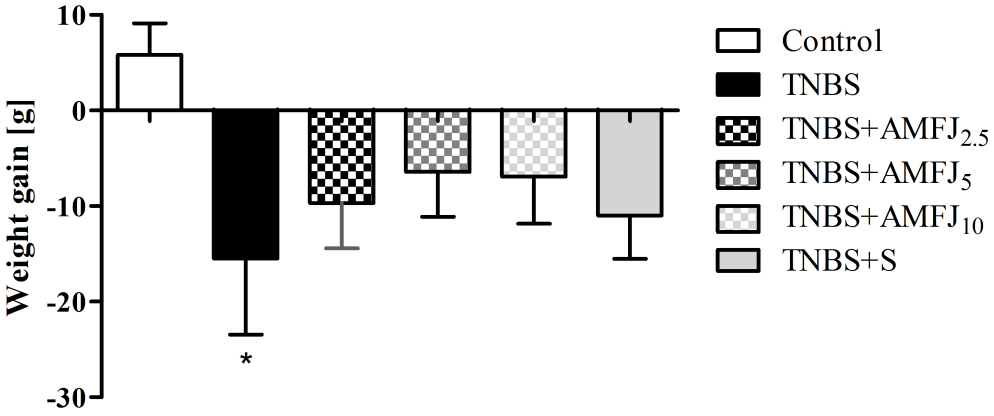
Fig. 3. Effect of *Aronia melanocarpa* fruit juice (AMFJ) at doses of 2.5, 5 and 10 mL/kg and sulfasalazine (S) on macroscopic indices of colonic damage in a rat 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model; *p<0.05 vs. Control

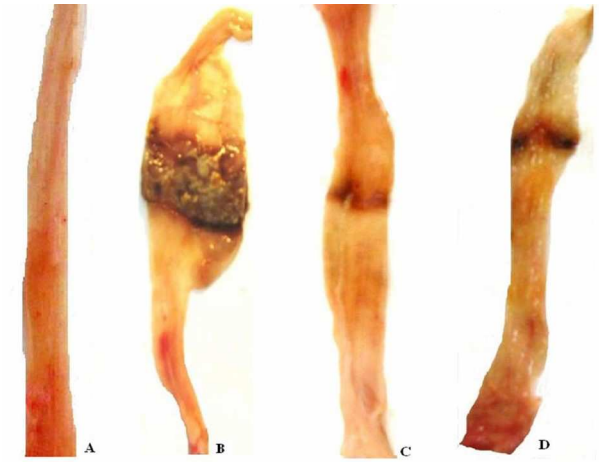
Fig. 4. Microscopic appearance of colons in a rat 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model: A. Control – Normal intestinal wall; B. TNBS – Diffuse destruction of intestinal wall; C. TNBS+$AMFJ_{10}$ – Zonal destruction of epithelial surface and inflammatory cell infiltration involving the muscularis mucosae; D. TNBS+S – Zonal destruction of epithelial surface and inflammatory cell infiltration involving the muscularis propria. H & E staining; magnification x 100

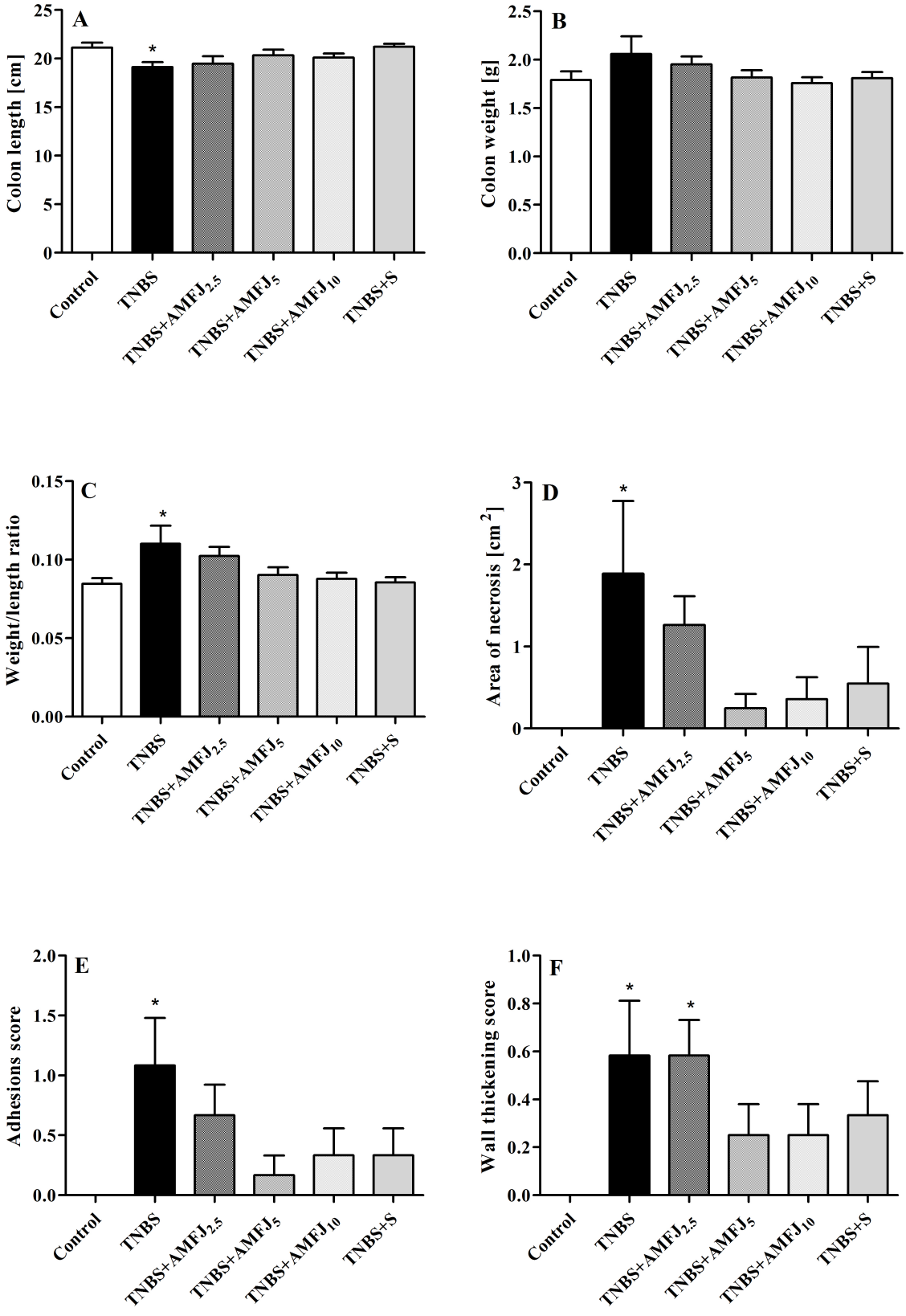
Fig 5. Effect of *Aronia melanocarpa* fruit juice (AMFJ) at doses of 2.5, 5 and 10 mL/kg and sulfasalazine (S) on microscopic scores of colonic damage in a rat 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model; $^{\&}p<0.05$, $^{\&\&}p<0.01$ vs. TNBS

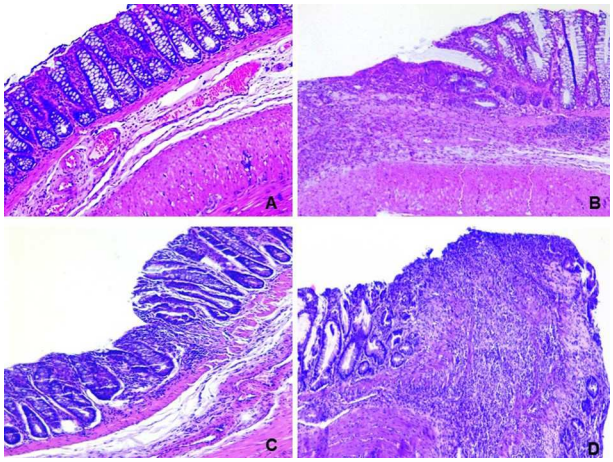
Fig. 6. Effect of *Aronia melanocarpa* fruit juice (AMFJ) at doses of 2.5, 5 and 10 mL/kg and sulfasalazine (S) on the level of thiobarbituric acid reactive substances (TBARS) in rat colon

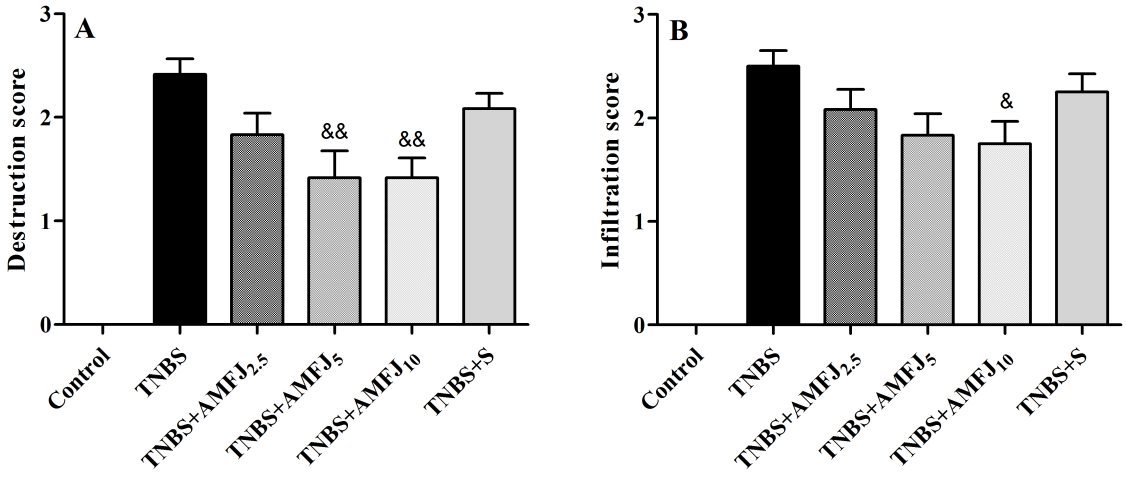
(panel A) and serum (panel B) in a 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model; *$p<0.05$ vs. Control

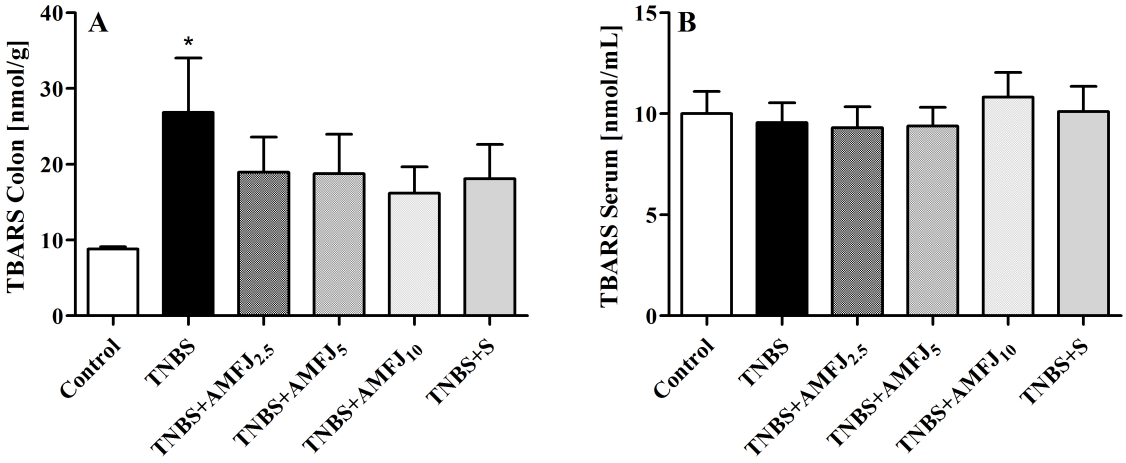





A
Colon length [cm]
25
20
15
10
5
0
*
Control
TNBS
TNBS+AMFJ2.5
TNBS+AMFJ5
TNBS+AMFJ10
TNBS+S
B
Colon weight [g]
2.5
2.0
1.5
1.0
0.5
0.0
C
Weight/length ratio
0.15
0.10
0.05
0.00
D
Area of necrosis [cm 2]
3
2
1
0
E
Adhesions score
2.0
1.5
1.0
0.5
0.0
F
Wall thickening score
1.0
0.8
0.6
0.4
0.2
0.0







Highlights:

- 2,4,6-trinitrobenzensulfonic acid (TNBS) induces severe colonic damage in rats.
- *Aronia melanocarpa* fruit juice (AMFJ) ameliorates TNBS-induced colitis.
- AMFJ alleviates oxidative stress in rat colons in TNBS-induced colitis.
- The effect of AMFJ is comparable to or even higher than that of sulfasalazine.