https://doi.org/10.31925/farmacia.2022.3.10 **ORIGINAL ARTICLE**

COMPARATIVE STUDY ON THE PROTECTIVE EFFECTS OF *NIGELLA SATIVA* OIL, CURCUMIN, AND HYDROXYTYROSOL AGAINST DEXTRAN SULPHATE SODIUM-INDUCED COLITIS IN MICE

RUMYANA SIMEONOVA [1]*, DIMITRINA ZHELEVA-DIMITROVA [2], NIKO BENBASSAT [2], IONKO SAVOV [3], RENETA GEVRENOVA [2]

[1]*Department of Pharmacology, Pharmacotherapy and Toxicology, Faculty of Pharmacy, Medical University of Sofia, Bulgaria*
[2]*Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Sofia, Bulgaria*
[3]*Institute of Emergency Medicine "N. I. Pirogov", Bul. Totleben 21, Sofia-1000, Bulgaria*

**corresponding author: rsimeonova@pharmfac.mu-sofia.bg*

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Abstract

The present study compared the protective effects of three commonly used plant-derived substances and products on dextran-induced colitis in mice. High-resolution gas chromatography-mass spectrometry (GC-HRMS) was used to identify the *Nigella sativa* oil (NSO) composition. The colitis was induced by a 7-day oral dextran 3% administration. The levels of NOx, CRP, MDA, GSH, IL-6, and the enzymatic activity of CAT, SOD, GPX and MPO were measured. The palmitic, linoleic, and oleic acids were detected in NSO. The colitis was evaluated by increased disease activity index, level of inflammatory markers, and disturbed oxidative stress markers. NSO, curcumin, and hydroxytyrosol markedly attenuated colonic inflammation by inhibiting MPO activity and decreasing the augmented levels of IL-6, NO and CRP. These compounds improved colon antioxidant defence mechanisms by reducing MDA quantity and increasing the level of GSH and antioxidant enzymatic activities. The investigated compounds ameliorated colon injury and inflammatory signs as visualized by histopathological examination. The results revealed that all three natural substances have similar colon protective effects.

Rezumat

Studiul a urmărit efectele protectoare a trei substanțe și produse derivate din plante, asupra colitei induse la șoareci, cu dextran. Compoziția uleiului de *Nigella sativa* (NSO) a fost identificată prin GC-HRMS. Colita a fost indusă prin administrarea orală de dextran 3% timp de 7 zile. Au fost determinate concentrațiile de NOx, CRP, MDA, GSH, IL-6, precum și activitatea enzimatică a CAT, SOD, GPX și MPO. Acizii palmitic, linoleic și oleic au fost identificați în NSO. În cadrul modelului experimental s-a determinat creșterea indicelui de activitate a bolii, nivelul markerilor inflamatorii și a markerilor de stres oxidativ. NSO, curcumina și hidroxitirozolul au atenuat semnificativ inflamația colonului prin inhibarea activității MPO și scăderea nivelurilor crescute de IL-6, NO și CRP. Acești compuși au optimizat mecanismele de apărare antioxidantă ale colonului prin reducerea cantității de MDA și creșterea nivelului activității GSH și a enzimelor antioxidante. Conform analizelor histopatologice, compușii investigați au ameliorat leziunile colonului și parametrii inflamatorii. Rezultatele au arătat că toate cele trei substanțe naturale au efecte similare, protectoare, asupra colonului.

Keywords: DSS-induced colitis, *Nigella sativa*, curcumin, hydroxytyrosol

Introduction

Ulcerative colitis (UC) is an inflammatory bowel disease (IBD) with an increasing prevalence in developed countries. In the seven major markets (7MM: US, France, Germany, Italy, Spain, UK and Japan), the diagnosed incident cases of UC are expected to increase from 94,303 cases in 2016 to 108,428 cases in 2026 at an Annual Growth Rate (AGR) of 1.5% [22].

The pathogenesis of the disease is not well elucidated, but it is known that it involves oxidative stress, inflammation, and finally, immune dysregulation. It is well established that increased levels of reactive oxygen species (ROS), pro-inflammatory cytokines and neutrophils play a crucial role in decreased colon immunity [38].

Typical symptoms include bloody diarrhoea, abdominal pain, urgency to defecate, weight loss, etc. Currently, prescribed drugs maintain remission, decrease the risk of complications and improve the quality of life. The treatment includes 5-aminosalicylates, corticosteroids for severe disease, followed by a transition to steroid-sparing agents with a thiopurine, anti-tumour necrosis factor agents, or adhesion molecule inhibitors [14]. The risk of disease complications and adverse drug reactions could be alleviated by dietary support with the addition of food supplements with ulcer-protective properties and a well-established safety profile.

In recent years, a large number of studies have been conducted on the ulcer protective effects of many biologically active substances as curcumin, a phenolic natural product isolated from the rhizome of *Curcuma longa* (turmeric), which has been used for centuries in China and Southeast Asia [29], glabridin, an isoflavonoid from *Glycyrrhiza glabra* [26], quercetin [11] and hesperidin [45], flavonoids in citrus fruits, hydroxytyrosol, a polyphenolic compound from extra virgin olive oil (EVOO) etc. [37, 38]. Extracts from *Camellia sinensis* [31], *Crataegi fructus* [15], *Zingiber officinale* [12], *Nigella sativa* [2, 13, 23, 27], *Panax notoginseng* [44] etc. have also been investigated for their protective effects on the gastrointestinal tract. Pharmacological mechanisms of action of these plant products have been elucidated. The essential mechanisms are: reduction in colonic myeloperoxidase (MPO) activity and tumour necrosis factor (TNF)-α production [31], decreased inflammation and improved leukotriene B4 levels [15], inhibition of ICAM-1, nitric oxide (NO) production and inducible NO synthase (iNOS) gene expression [26], COX2 inhibition [41] etc.

The present study aimed to compare the mucosal protective effects of three of the most discussed substances, curcumin (CCN) and hydroxytyrosol (HT), and the vegetable product - *Nigella sativa* oil (NSO) against DSS induced colitis in mice. CCN was chosen because of its proven anti-inflammatory effects [3, 19, 28, 41], HT as a potent antioxidant molecule [37, 38], and NSO or Black seed oil, for its widespread use as a plant-derived medicine [2, 16, 17, 40] or as a spice in Middle Eastern and Indian cuisine. The primary fatty acid found in some species of *Nigella sativa* seeds was linoleic acid (C18:2), an essential fatty acid which cannot be biosynthesized by mammalian cells. Linoleic acid has great importance in human life due to its vital contribution to the biosynthesis of polyunsaturated fatty acids with long chains (C20:5; C22:5; C22:6) and the synthesis of prostaglandins (PG1, PG2 and PG3) [40].

Black cumin seeds have been valued for many pharmacological actions, such as antidiabetic, anti-cancer, immunity modulator, analgesic, antimicrobial, anti-inflammatory, spasmolytic, bronchodilator, anti-oxidant etc. [2, 16, 17]. Therapeutic properties of *Nigella sativa* have been attributed to thymoquinone, considered the most significant bioactive component of the essential oil [16, 17]. *Nigella sativa* is commonly added to food as seeds or essential oil for various beneficial effects on the overall quality of different foods and especially for inhibiting the growth of food-borne pathogens [17].

Materials and Methods

Chemicals

Bovine serum albumin (fraction V), beta-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NADPH), cumene hydroperoxide, glutathione reductase (GR), hydroxytyrosol, oxidized glutathione (GSSG), reduced glutathione (GSH), 2-thiobarbituric acid (TBA) and trichloroacetic acid (TCA), were purchased from Sigma Chemical Co. (Taufkirchen, Germany). 2,2-Dinitro-5,5 dithiodibenzoic acid (DTNB) was obtained from Merck (Darmstadt, Germany). Dextran Sulphate Sodium (DSS) salt (M.W 36 - 50 kDa, Ref = 160110) was from MP Biomedicals. Highly soluble curcumin under the trade name Cursol® was kindly donated by BioXtract (Les Isnes, Belgium). Ketamine and xylazine were purchased from a veterinary pharmacy in Sofia, Bulgaria. Trimethylsulpfnium hydroxide (0.25 M in methanol for GC derivatization) was obtained from Sigma-Aldrich. The reagents were of analytical grade.

Plant material

Nigella sativa seeds were purchased from a herbal pharmacy in Sofia, Bulgaria. The plant material (1 kg) was ground and sieved. The oil was obtained by pressing the seeds of black cumin. 100 mg of the oil was dissolved in 5 mL of diethyl ether. 50 µL of dimethyl sulphonium hydroxide was added to 100 µL of this solution. The mixture was injected directly with the injector temperature of at least 250°C. Pure *Nigella sativa* oil was used for the other experiments, and curcumin and hydroxytyrosol were dispersed in virgin olive oil.

Gas chromatography-high resolution mass spectrometry (GC-HRMS)

GC-HRMS analysis of the obtained oil was performed by gas chromatographic system Trace 1310 GC, Exactive Orbitrap GC-MS system (Thermo Fischer Scientific, Bremen, Germany). The data were preceded by Excalibur Software (Thermo Fischer Sci.). The identification of the compounds was performed by comparing their mass spectra and Kovats Indexes (RI) with those of the NIST 05 databases and literature data. The measured mass spectra were taken from the Automated Mass Spectral Deconvolution and the Identification System (AMDIS) before comparison with the databases. The spectra of the individual components were then transferred to the NIST Mass Spectral Search Program MS Search 2.0, where they were compared to NIST Mass Spectral Library reference compounds.

Animals

Thirty ICR female mice (22 - 24 g, 6 weeks old) were purchased from the National Breeding Centre, Sofia, Bulgaria. The mice were housed in plexiglass cages (6 *per* cage) in a 12/12 light/dark cycle under standard laboratory conditions (ambient temperature 20 ± 2°C and humidity 72 ± 4%) with free access to water and normal pelleted food, suitable for their age and produced according to ISO 9001:2008. One week of acclimatization was allowed before starting the study. All the performed procedures were approved by the Bulgarian Food Safety Agency (BFSA) (permissions № 208 with a protocol № 124/ 05. 10. 2018).

Experimental design

The animals were divided into 6 groups (Figure 1), with six animals in each group as follows: Group 1: control group, treated orally with virgin olive oil 0.1 mL/10 g bw; Group 2: DSS treated group (DSS 3.0% in the drinking water) [5]; Group 3: DSS + HT (HT at a dose of 50 mg/kg bw, dispersed in olive oil) [18]; Group 4: DSS + CCN (CCN at a dose of 50 mg/kg bw, dispersed in olive oil) [29]; Group 5: DSS + NSO (NSO, 2.0 mL/kg bw) [21]; Group 6: DSS + COMB (combination of HT and CCN, dispersed in NSO).

Excluding the control group, colitis was induced by oral administration of 3% DSS for 7 days in all other 5 groups. The tested compounds, at the above-mentioned doses, were administered orally for 14 consecutive days - 7 days simultaneously with DSS and one week after.

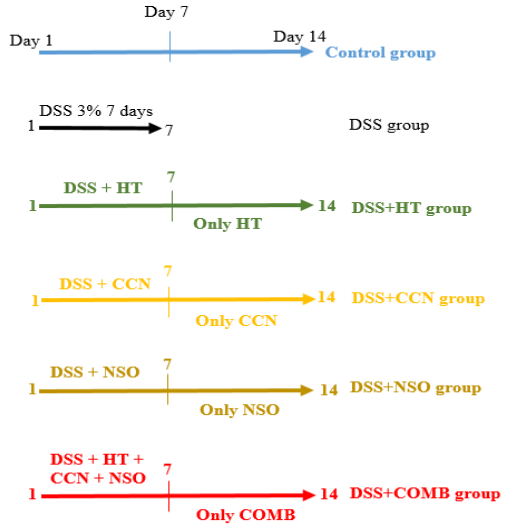

Figure 1.
Experimental design

During the experimental period, the weight of each animal, stool consistency and faecal blood were checked daily. The disease activity index (DAI) was scored using a scoring system described by Sánchez-Fidalgo *et al*. [37, 38].

DAI was determined by combining the scores for bodyweight loss, stool consistency and gross bleeding, divided by 3. The score was determined as follows: change in body weight loss (0: none, 1: 1 - 5%, 2: 6 - 10%, 3: > 11%), bleeding (0: negative, 1: slight bleeding, 2: moderate bleeding 3: severe bleeding), stool consistency (0: regular, 1: soft unformed excrement, 2: loose stool, 3: watery diarrhoea).

On the 15th day, after overnight starvation, the animals from all groups were sacrificed after anaesthesia with ketamine/xylazine (80 mg/10 kg bw, intraperitoneally), blood was sampled, and colons were removed from the appendix to the anus for macroscopic, histological and biochemical analysis. After the measurement of length and weight, the colons were cut and washed with physiological saline. A portion of the colons was used for biochemical evaluation, and the remaining parallel part of the colons was fixed in 10% neutral-buffered formalin for histological analyses.

Experimental methods

Oxidative damage was determined by measuring the quantity of thiobarbituric acid reactive substances (TBARS), expressed as malondialdehyde (MDA) equivalents as described by Polizio and Peña [21]. The reduced glutathione (GSH) was assessed by measuring the non-protein sulfhydryls after the precipitation of proteins with trichloracetic acid (TCA), using the method described by Bump [8]. The antioxidant enzymes activity was measured in the supernatant of 10% homogenates, prepared in 0.05M phosphate buffer (pH = 7.4). The glutathione peroxidase (GPx) was measured by NADPH oxidation using a coupled reaction system consisting of GSH, glutathione reductase (GR), and cumene hydroperoxide [39]. The catalase (CAT) activity was determined by measuring the decrease in absorbance at 240 nm of a reaction mixture consisting of H_2O_2 in phosphate buffer, pH = 7.0, and the requisite volume of the supernatant sample [1]. The superoxide dismutase activity (SOD) was measured according to the method of Misura and Fridovich [32]. The myeloperoxidase activity was assessed by the modified method of Sánchez-Fidalgo *et al*. [37]. The protein content was measured according to the method of Lowry *et al*. [30]. The levels of nitric oxides (NOx) were measured by the simple reaction of Griess, described by Rios *et al*. [35]. CRP was measured through a turbidimetric quantitative test using commercially available kits for biochemical analyser Mindray - BP 120 (China) as described in manufacturer instructions. The interleukin 6 (IL-6) level in the serum was analysed using electro-chemiluminescence immunoassay "ECLIA", with kits for immunoassay analyser Cobas - Roche, Basel, Switzerland.

Histopathological examination

Histopathological examination was performed using the method of Bancroft and Gamble [4]. The sections were observed under a high power microscope, and photomicrographs were taken using Olympus CX31 and Camera Olympus x Optical zoom with objective PlanaC 4/0.10 (Karl Zeiss, Germany).

Statistical analysis

Statistical software MEDCALC was used for the analysis of the data. The results are expressed as mean ± SD of six mice in each group. The significance of the data was assessed using the nonparametric Mann-Whitney U test. The values of $p \leq 0.05$ were considered statistically significant.

Results and Discussion

Phytochemical composition of NSO

The chemical composition of NSO was determined by GC-HRMS, and the results are presented in Table I.

The main compounds in the tested NSO were palmitic, linoleic and oleic acid. The phytochemical profile of the NSO from this study confirms the data obtained by Toma *et al*. [40]. Using Gas Liquid Chromatography (GLC), the authors had found that the primary fatty acid of the Tunisian NSO was the linoleic acid (C18:2), representing about 63.71% of the total fatty acids.

General assessment

In this study, DSS-induced colitis is characterized by an increase of DAI established by the weight loss, faecal consistency, and the presence of blood in the stool (haematochezia). The body and colon weight loss is a significant marker to evaluate disease aggravation in DSS-induced colitis. The chemical led to a statistically significant ($p < 0.05$) body weight loss, loose stools and bloody diarrhoea, especially after the 3rd day of the beginning of the experimental period compared with the control group value (Figure 2).

Table I

Chemical composition of NSO

№	tR (min)	Identified compounds
1	17.05	Tridecanoic acid
2	19.20	Palmitic acid
3	20.88	Linoleic acid
4	20.94	Oleic acid
5	21.12	Stearic acid
6	22.62	9-hexadecan-1-ol
7	26.90	Retinoic acid
8	40.20	Vit. D_2
9	44.14	Myristic acid
10	44.88	Margaric acid
11	46.49	Eicosanoic acid

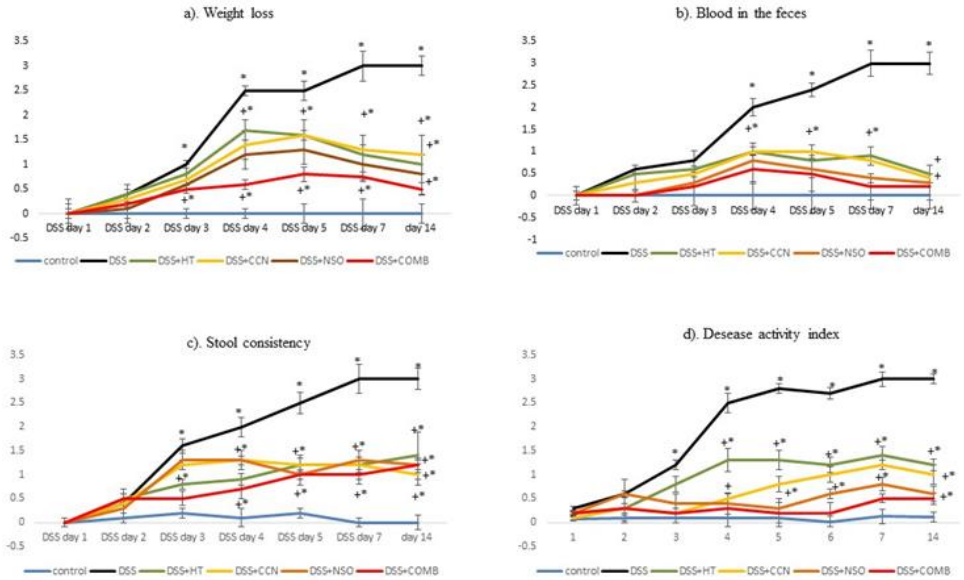

Figure 2.

Weight loss (a), blood in the faeces (b), stool consistency (c), DAI (d). *$p < 0.05$ *vs*. control; $^{+}p < 0.05$ *vs*. DSS

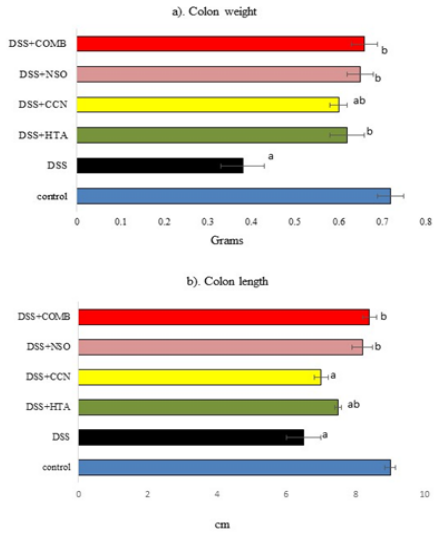

Figure 3.

Colon weight (a), length (b)

$^{a}p < 0.05$ *vs*. control; $^{b}p < 0.05$ *vs*. DSS

Body and colon weight loss and haemorrhage of the rectum are connected with colon shortening after DSS treatment (Figures 3a and 3b). Many authors reported that colon length is inversely linked with the gravity of DSS-induced colitis [36-38, 46]. The macroscopic differences in the large intestines were also visible. The administration of the tested compounds, alone or in combination, alleviated significantly ($p < 0.05$) all parameters involved in DAI scoring compared with the DSS group. However, these parameters, except bleeding, did not reach control levels by the 14th day.

Evaluation of antioxidant enzyme activity and GSH level

Effects of HT, CCN and NSO alone or in combination on enzymes' antioxidant activity and the level of GSH on DSS-treated mice are shown in Figure 4. In contrast to the data presented in other studies [34, 36], where the DSS-induced colitis provoked a decrease

in antioxidant enzymes activity, in the present research, DSS oral intake increased significantly ($p < 0.05$), the activity of SOD, CAT, and GPx in the colon tissues with 25%, 29% and 44% respectively and decreased the GSH content with 24% compared with the control group. Similar antioxidant enzymes activity augmentation was observed in patients with UC [25]. ROS are potent tissue-injuring molecules in all inflammatory diseases, including the UC [25]. The antioxidant enzymes in the colon mucosa are involved in the neutralization and resistance to the toxic effects of ROS, because of which their activity could be increased. The first line of defence is the SOD enzyme family that converts the superoxide anion (O2-) into the stable and easily diffusible hydrogen peroxide (H2O2) that is neutralized to water by CAT or GPx.

Compared to the DSS group, the administration of HT, a well-known powerful phenolic antioxidant compound [38] from *Olea europaea*, produced an additional significant ($p < 0.05$) increase in SOD activity by 21%, GPx activity by 26%, and GSH level by 44%. Many studies described the beneficial effect of HT on UC [37, 38] and many other inflammatory diseases [20].

The curcumin, administered as a highly soluble form of Cursol®, did not change the activity of the antioxidant enzymes significantly, but increased the GSH content by 33% compared with the DSS group. The most pronounced antioxidant effects were seen in the NSO treated group and in the combination group. The results from the phytochemical evaluation showed that NSO is a rich source of unsaturated fatty acids such as palmitic acid, linoleic acid, oleic acid, stearic acid etc. The NSO raised SOD, CAT, GPx activity and GSH levels by 21%, 32%, 23% and 40%, respectively, compared to the DSS group. This is probably due to the content of the above-mentioned organic acids. The combination group treatment showed the highest pronounced antioxidant effects. In this group, SOD, CAT, GPx activity and GSH levels were increased by 31%, 36%, 39% and 62%, respectively, compared to the colitis-induced group (Figure 4).

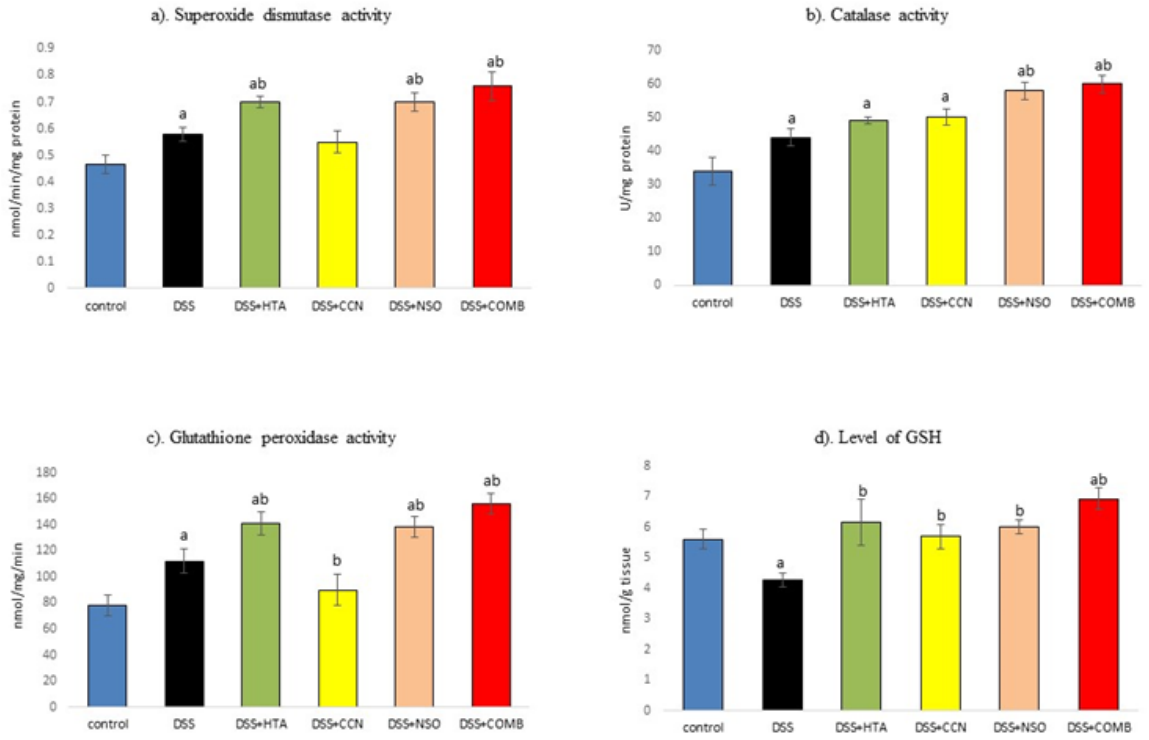

Figure 4.
Antioxidant enzymes activity in the colons of experimental groups. SOD (a), catalase (b), GPx (c), and GSH level (d); [a]$p < 0.05$ *vs.* control; [b]$p < 0.05$ *vs.* DSS

Level of pro-inflammatory markers

When the formation of H_2O_2 exceeds its removal, alternative reactions with the appearance of hydroxyl radical (OH-) through the Fenton and Haber–Weiss reactions or the formation of hypochlorous acid (HOCl) *via* neutrophil-derived myeloperoxidase (MPO) activation are initiated [24]. The myeloperoxidase (MPO) is a major enzyme of neutrophils. It is also present in monocytes and macrophages. Thus, the MPO activity reflects the degree of neutrophil infiltration, so that it can serve as a marker of acute inflammation [24]. The present study demonstrated a significant increase of MPO activity (Figure 5a) by 87% in the DSS group compared with the control mice, which is a sign of increased ROS formation in this pathology and consequent inflammation. Due to the higher SOD activity and

H_2O_2 levels, respectively, the MPO activity was also increased since this enzyme uses H_2O_2 as a substrate. The administration of HT, CCN, NSO and their combination in colitis induced mice decreased significantly ($p < 0.05$) the activity of this enzyme by 29%, 43%, 57% and 39%, respectively, compared to the DSS group (Figure 5a). Our data confirmed the results obtained by many research teams who have evaluated the ulcer protective effects of these substances [23, 29, 37, 38].

MDA is an end product of lipid peroxidation. It is considered very harmful and responsible for the release of cell contents and cell death, causing tissue and organ damage [34]. The levels of MDA in the experimental groups are presented in Figure 5b. The MDA quantity was significantly ($p < 0.05$) elevated by 42% in the colons of DSS mice compared to the control group. Similar results were reported by other researchers [34]. In the present study, it was observed a significant ($p < 0.05$) decline in the levels of MDA, with approximately 27%, upon treatment with HT, CCN, and NSO. It has been reported that hydroxytyrosol supplementation inhibits oxidative damage, suppresses lipid peroxidation and modulates antioxidant enzymes in human peripheral blood lymphocytes [6]. In the intestinal mucosa, curcumin reduces levels of ROS, such as NO, superoxide anions and MDA [9]. It has been reported a significant reduction of MDA levels in TNBS-induced experimental colitis in rats [23].

The same effect on the MDA levels was found after 6 weeks of *Nigella sativa* powder consumption in patients with UC [33]. The combination of the three compounds reduced the MDA levels by 53%, compared to the DSS group.

Interleukin 6 acts as both a pro-inflammatory cytokine and an anti-inflammatory myokine, and its levels are increased in necrotizing colitis (NEC) [9]. The plasma IL-6 is significantly associated with higher NEC morbidity and mortality [9]. In the present experiment, IL-6 was significantly ($p < 0.05$) increased in the serum of DSS injured mice by 120% compared to the control group (Figure 5c). NSO and the combination of the 3 compounds decreased in a statistically significant manner ($p < 0.05$) the serum level of this pro-inflammatory cytokine, by approximately 35%, compared to the DSS group. IL-6 reducing effect of *Nigella sativa* has been described by some authors [7, 23].

C-reactive protein (CRP) is a marker of inflammation and is produced almost exclusively by hepatocytes. The central stimulus for its production is IL-6 [43]. CRP is a valuable marker for detecting and following up on Crohn's disease activity (CD) in humans. Still, UC has only a modest CRP response despite active inflammation, and the reason for this is unknown [43]. In the study of Do and Woo [10], CRP was upregulated in mice serum from the third day of DSS treatment. In the present research, all DSS-treated groups showed a higher serum CRP level compared to the control group. The treatment with the investigated compounds did not decrease it effectively compared with the DSS group (Figure 5d).

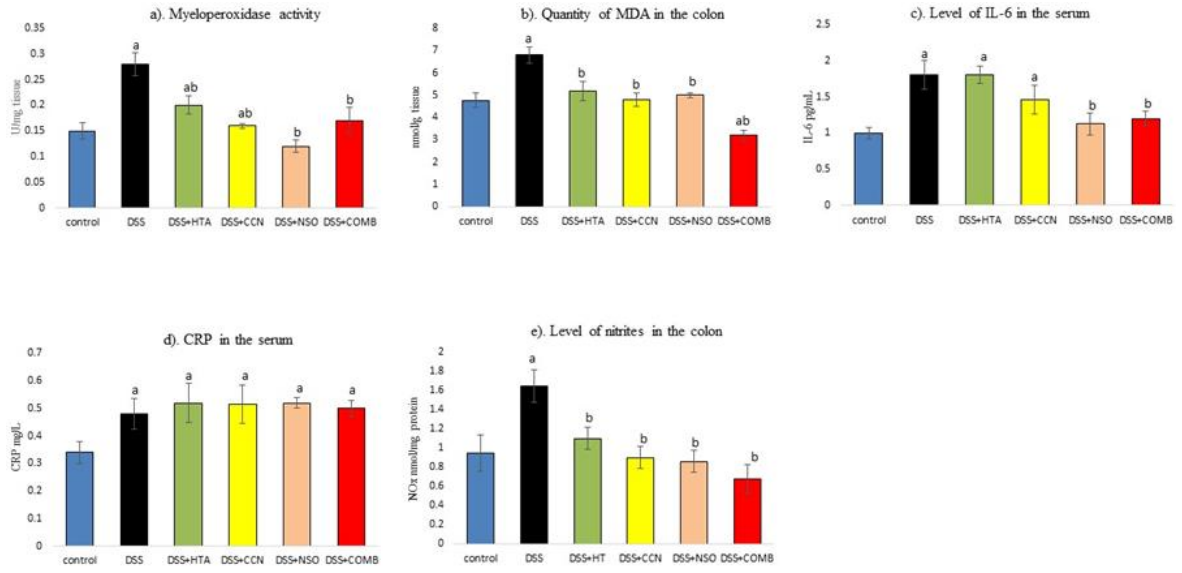

Figure 5.

Levels of the proinflammatory markers. Colon MPO activity (a), MDA quantity in the colon (b), serum IL-6 level (c), CRP level (d); NOx level in the colon (e); [a]$p < 0.05$ *vs.* control; [b]$p < 0.05$ *vs.* DSS

Physiological levels of nitric oxide protect the intestinal mucosa. Still, the large amounts of NO released via inducible nitric oxide synthase (iNOS) during intestinal inflammatory disease progression can lead to tissue injury and necrosis [9]. During inflammatory events, iNOS produces nitric oxide in pathogenic amounts. In intestinal inflammatory diseases, chronic iNOS stimulation likely leads to the breakdown of the intestinal

integrity due to the generation of reactive nitrogen species (RNS) [9]. We found an increase of 74% ($p < 0.05$) in colon NO level in the DSS group compared to the control group. Treatment with HT, CCN, NSO, and the combination group reduced the NO level by 33%, 45%, 48%, 59%, respectively, compared to the DSS group. For all three compounds, the NO reducing effect in colitis models has been described [2, 9, 37].

Histological assessment

The histological characteristics of the colons were assessed through well-known H&E staining, and the results are presented in Figure 6. In the control group, the colons were with normal morphology and well-formed mucosa without changes of crypts, no signs of mucosal thickening, and ulcerations were not seen (Figure 6a). However, the DSS induced mice presented severe epithelial damage with partial mucosa with inflammatory changes and focal lesions, mucosa thickening and destruction of the architecture (Figure 6b). It has been reported that the DSS-induced colitis model can represent several histopathological features of UC, such as mucosal erosion, loss of intestinal crypts, and ulceration. The treatment with HT and CCN relatively preserved the mucosa with moderately pronounced inflammatory changes and poor response to the lymphatic apparatus (Figure 6b) compared with the colons from mice treated with DSS. In the present study, NSO and the combination of 3 compounds significantly ameliorated histological structure. The mucosa showed moderate inflammatory changes and focal submucosal inflammatory infiltrates compared with the DSS-induced colitis model (Figure 6e, Figure 6f).

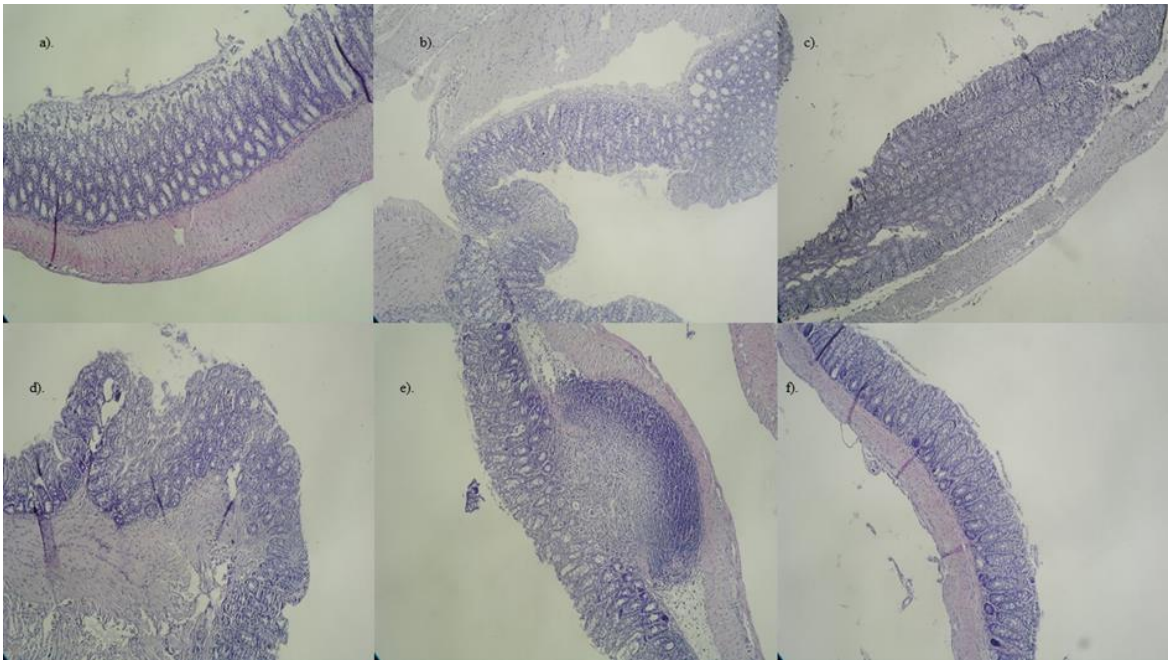

Figure 6.
Histological assessment of the colon. Control group (a), DSS-induced group (b), DSS + HT group (c), DSS + CCN group (d), DSS + NSO group (e), DSS + COMB group (f).

Conclusions

All studied biologically active substances show a pronounced ulcer protective effect, but this effect is most observed in animals treated with *Nigella sativa* oil and in the group treated with the combination of the three substances. The disadvantage of this study is the lack of positive control, a drug established in the treatment of ulcerative colitis such as salazopyrin or mesalazine. This drawback requires new research in the future with other naturally derived products that could be compared with the conventional treatment of UC.

Acknowledgement

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Conflict of interest

The authors declare no conflict of interest.

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