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Anti-inflammatory and immunomodulatory effects of *Spirulina platensis* in comparison to *Dunaliella salina* in acetic acid-induced rat experimental colitis

Mohamed M. Abdel-Daim¹, Sameh M. Farouk², Fedekar F. Madkour³, and Samar S. Azab⁴

¹Pharmacology Department, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt, ²Cytology & Histology Department, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt, ³Marine Science Department, Faculty of Science, Port Said University, Port Said, Egypt, and ⁴Pharmacology & Toxicology Department, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt

Abstract

**Context:** *Spirulina platensis* (SP) is used as a source of protein and vitamin supplement in humans without any significant side-effects. *Dunaliella salina* (DS) is also regarded as one of the richest natural producers of carotenoid, thus used as a source of antioxidants to protect cells from oxidative damage. Spontaneous or bacterial-induced inflammatory response and altered immune response driven by microbial factors in the enteric environment are proposed to be involved in its pathological events. For instance, the mucosal immune system is reported as cytotoxic reactive oxygen species (ROS) in addition to free radicals, both the nitric oxide (NO) system and cycloxygenase (COX-2) have been shown to modulate many events in the gastrointestinal tract. Several studies are reporting that both inducible nitric oxide synthase (iNOS) and COX-2 have been shown to modulate many events in the gastrointestinal tract. Studies are reporting that both inducible nitric oxide synthase (iNOS) and COX-2 have been shown to modulate many events in the gastrointestinal tract. Studies are reporting that both inducible nitric oxide synthase (iNOS) and COX-2 have been shown to modulate many events in the gastrointestinal tract.

**Materials and methods:** *Spirulina* and *Dunaliella* were investigated at the same dose of 500 mg/kg body weight for their modulatory effect against acetic-acid induced ulcerative colitis (UC) in rats. The colonic lesion was analyzed by examining macroscopic damage, bloody diarrhea scores, colon weight/length and change in body weight of tested rats. Colon lipid peroxidation and oxidative stress markers were examined by evaluating malondialdehyde (MDA), protein carbonyl (PCO), catalase (CAT), reduced glutathione (GSH) and superoxide dismutase (SOD). Colon inflammatory markers; myeloperoxidase (MPO) and prostaglandin (PGE₂) as well as proinflammatory cytokines; tumor necrosis factor (TNF-α) and interleukins (IL-1β, IL-6) were also studied.

**Results:** The colonic mucosal injury, biochemical and histopathologic results suggest that both SP and DS exhibit significant modulatory effect on acetic acid-induced colitis in rats, which may be due to a significant increase of antioxidant enzymes activity and significant inhibition of lipid peroxidation and inflammation markers. Results showed that in comparison to Sulfasalazine, SP exhibited better therapeutic and safety profile than DS against acetic acid-induced UC.

**Discussion:** Results showed that in comparison to Sulfasalazine, SP exhibited better therapeutic and safety profile than DS against acetic acid-induced UC.

**Conclusion:** This study suggests potential benefits of SP and DS in an experimental model of colitis.

Introduction

Ulcerative colitis (UC) and Crohn’s disease (CD) are immunologically mediated disorders that are collectively referred to as inflammatory bowel diseases (IBD). Ulcerative colitis affects primarily the mucosal lining of the colon and rectum, whereas CD may involve any segment of the gastrointestinal tract. Etiology of IBD remains obscure, although environmental factors, in combination with genetic factors and altered immune response driven by microbial factors in the enteric environment are proposed to be involved in its pathological events. For instance, the mucosal immune system is reported as the main mediator of intestinal inflammation and injury, with cytokines playing a central role in initiating inflammation. The activation of the intestinal immune system results in the production of proinflammatory cytokines, such as tumor necrosis factor (TNF-α) and interleukin-1β (IL-1β), prostaglandins (PG) and leukotrienes (LT).

Importantly, infiltration of inflammatory cells, such as neutrophils, in addition to the overproduction of proinflammatory cytokines ultimately gives rise to mucosal disruption and ulceration. Neutrophils' infiltration, assessed by myeloperoxidase (MPO), is a key source of production of the cytotoxic reactive oxygen species (ROS).

In addition to free radicals, both the nitric oxide (NO) system and cycloxygenase (COX-2) have been shown to modulate many events in the gastrointestinal tract. Several studies are reporting that both inducible nitric oxide synthase (iNOS) and COX-2 are evidently upregulated after the stimulation of host cells with bacteria or inflammatory cytokines, such as TNF-α and IL-1, respectively.
indicating their role in exacerbation of the underlying ulcerative pathogenesis \textsuperscript{10–12}.

Currently available therapies for IBD are only effective in ameliorating the disease symptoms, while having many concomitant disadvantages \textsuperscript{13}. In this context, a number of recent studies have renewed interest in the antioxidant potential of \textit{Spirulina platensis} (SP) and \textit{Dunaliella salina} (DS) for the management of inflammatory conditions and oxidative damages \textsuperscript{14–16}. \textit{Spirulina} (SP) refers to the dried biomass of \textit{Arthospira platensis}, an oxygenic photosynthetic microscopic cyanobacterium found worldwide in fresh and marine waters. This alga has been used as a source of protein and vitamin supplement in humans without any significant side-effects. Apart from its high (up to 70\%) content of protein, it also contains vitamins, especially B12 and provitamin A (\(\beta\)-carotenes), and minerals, especially iron. It is also rich in phenolic acids, tocopherols and \(\gamma\)-linolenic acid \textsuperscript{17}. C-phycocyanin (C-PC), one of the major biliproteins of SP, is reported to exhibit an antioxidant, radical scavenging properties, as well as selective cyclooxygenase-2 inhibition, anti-inflammatory and anticancer effects \textsuperscript{18}.

On the other hand, DS is a unicellular marine phytoplankton that belongs to the phylum Chlorophyta. \textit{Dunaliella} is regarded as food supplement as it is one of the richest natural producers of carotenoid, producing up to 15\% of its dry weight under suitable conditions. Therefore, DS could be used as hepatoprotective, antioxidant, free radical scavenger, protect cells from oxidative damage \textsuperscript{16,19}. Until today, \(\beta\)-carotene remains the major natural product harvested from DS. \(\beta\)-carotene prevents cancer of various organs, including ovary, prostate, cervix, breast, pancreas, lungs, stomach, rectum and colon by antioxidant activity \textsuperscript{20}. It boosts immune response \textsuperscript{21}, inhibits neoplastic transformation and controls of growth \textsuperscript{22}.

To date, however, the possible modulatory role of either SP or DS in colon inflammation has not been yet verified; hence, we aimed in the current investigation to evaluate and compare the possible modulating effect(s) of SP and DS on acetic acid-induced ulcerative colitis model in rats.

Materials and methods

Chemicals

Pure premium SP powder was purchased from (HerbaForce, Berkshire, UK). \textit{Dunaliella salina} was collected from a highly saline concentrating pond at the solar saltern of Port Fouad, Port Said, Egypt, during summer 2010. The collected DS was examined, microscopically identified, cultivated and harvested by centrifugation at 3500 rpm for 15 min. The dried material was mixed with methanol and sonicated, then placed on the shaker platform for 24 h for cold extraction. The filtrate was evaporated by rotary evaporator at 30–35\°C and the mass obtained was dissolved in distilled water as a vehicle and employed for further experiments \textsuperscript{16}. Sulfasalazine (SSZ) was obtained from El-Kahira Pharmaceutical Company, Cairo, Egypt. MDA, GSH and SOD kits were purchased from Biodiagnostic Co., Giza, Egypt. Acetic acid was obtained from El-Nasr Chemical Co. (Cairo, Egypt). IL-1, IL-6, PGE2 and TNF-\(\alpha\) ELISA kits were purchased from R&D Systems GmbH, Wiesbaden, Germany. All other chemicals used were of analytical grade.

Animals

Forty male Wistar albino rats, with a mean weight of 150–170 g were obtained from the animal house of the National Central Institute; Dokki, Cairo, Egypt, and allowed to acclimatize to their environment for 1 week before the experiment. The rats were housed in stainless-steel cages (eight animals per cage) and kept on an equal light and dark cycle and constant environmental conditions. The rats were allowed to free access to water and food (fed on standard pellet). All efforts were made to minimize animal pain or suffering during experimentation. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in the European Community Guidelines (EEC Directive of 1986; 86/609/EEC) and was approved by the Animal Care and Use Committee of Faculty of Veterinary Medicine, Suez Canal University, Cairo, Egypt (Approval no; 20146).

Study groups

Animals were divided into five groups (eight rats each). The first group received vehicle (oral saline) for 15 days followed by single rectal instillation of saline on day 16 and served as normal control. The second group (acetic acid; AA group) was given acetic acid intrarectally at day 16 as single dose and served as positive control (ulcerated, non-treated). The third group was given sulfasalazine 500 mg/kg orally for three consecutive days (13th, 14th and 15th) then subjected to rectal instillation of acetic acid, as in group 2 on day 16. Groups 4 and 5 were given orally SP and DS at 500 mg/kg, respectively, for 15 consecutive days, then also subjected to rectal instillation of acetic acid, as in group 2 on day 16. On the 17th day, 24 h after induction of colon ulceration, rats were sacrificed under anesthesia and laparotomy was performed. Sulfasalazine, SP and DS doses were determined from reports of previous work \textsuperscript{16,23,24}. Colonic segments were excised, freed of adherent adipose tissue, washed with saline, and were used for macroscopic scoring, histopathological examination and biochemical studies. Colonic samples were stored immediately at \(-20\)\°C till analysis of oxidative stress, inflammatory and immunomodulatory markers.

Induction of colonic inflammation in rats

The animals were fasted for 24 h with access to water \textit{ad libitum} before induction of colitis. Induction of colitis was performed using a modification of the method described by Millar et al. \textsuperscript{25}. Each rat was sedated by an intraperitoneal injection of pentobarbitone (35 mg kg \(^{-1}\)). A solution of 1 ml (4\%, v/v) acetic acid (Merck, Germany) in saline was infused for 30 s using a polyethylene tube (2 mm in diameter), which was inserted through the rectum into the colon to a distance of 8 cm. The rats were then maintained in a supine Trendelenburg position for 30 s to prevent early leakage of the intracolonic instillate. The acetic acid was retained in the colon for 30 s after which the fluid was withdrawn \textsuperscript{26}.

Colitis-macroscopic scoring

Mucosal damage was assessed macroscopically at post-mortem laparotomy by the scoring system developed by
Millar et al. 25, where 6 cm of colon extending proximally for 2 cm above the anal orifice were cut, weighed then split longitudinally. In each rat, the macroscopic injury of each ulcer was scored by an independent observer according to a scoring system described by Cao et al. 27 ranging from 0 to 5 as follows: (0) no macroscopic changes or observed damage; (1) localized hyperemia with no ulcers; (2) linear ulcers with no significant inflammation; (3) linear ulcers with inflammation at one site; (4) more ulcerative and inflammatory sites, the size of ulcers <1 cm and (5) multiple inflammations and ulcers, the size of ulcers ≥1 cm. Intermediate values reflected intermediate appearances. Furthermore, the condition of stool of the rats was evaluated one day before and after acetic acid treatment. The score for stool occult blood for each rat was determined as follows: (0: negative, 100: positive).

Assessment of oxidative stress and lipid peroxidation markers

The colon lipid peroxidation was evaluated in colon tissue homogenate by measurement of colonic MDA content according to Mihara and Uchiyama 28, and colonic protein carbonyl (PCO) was assessed according to Levine et al. 29. The carbonyl content was calculated in terms of nmol/mg protein. The non-enzymatic antioxidant biomarker; reduced glutathione (GSH) was assessed according to Beutler et al. 30. The enzymatic antioxidant biomarker: superoxide dismutase (SOD) was evaluated according to the study of Nishikimi et al. 31 and catalase (CAT) according to Aebl 32.

Evaluation of the inflammatory and immuno-modulatory markers

Myeloperoxidase (MPO) activity was used as an index of leukocyte adhesion and accumulation in several tissues, including the intestine. Estimation of MPO activity was carried out according to Krawisz et al. 33. The proinflammatory cytokines: IL-1β, IL-6, TNF-α and PGE2 were assessed using commercially available kits from R&D Systems GmbH, Wiesbaden, Germany and followed Reinecker et al.’s 34 method; and were quantified by enzyme-linked immunoabsorbent assay [ELISA] (Amersham Pharmacia Biotech, Little Chalfont, UK) and the results were expressed as pg/g wet tissue. PGE2 was also measured by ELISA kits (R&D Systems, Minneapolis, MN) 35, and expressed as pg/g wet tissue.

Colitis-histopathological evaluation

The prepared colonic sample sections were stained using the following stains 36: Harris hematoxylin and eosin (H&E), periodic-acid Schiff (PAS) technique, Alcian blue technique (pH 2.5) and Masson’s trichrome. The stained sections were examined for the histopathological findings of colonic architectural changes. Representative photomicrographs were taken using Olympus BX41 research optical photomicroscope fitted with Olympus DP25 digital camera (Tokyo, Japan) in Cytology and Histology Department, Faculty of Veterinary Medicine, Suez Canal University.

Colitis-immunohistochemical examination

Immunohistochemical examination was carried out as previously described 37,38 using specific antibodies targeting COX-2 (Novus Biologicals NB100-689, Littleton, CO) and iNOS (Novus Biologicals NB1-50606, USA). Images were taken using Leica DM2500 microscope (Wetzlar, Germany) and analyzed using an image analyzer Leica Q win V3 program in the Histology Department, Faculty of Medicine, Ain Shams University. Area percentage of the immunohistochemical stains was assessed for five fields per slide in all the treatment groups.

Statistical analysis

All data are expressed as mean ± standard error of the mean (S.E.M.) of eight rats per experimental group. Statistical analysis was performed using Instat 3.06 statistical software package (San Diego, CA). Parametric one-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparisons test was used to compare the mean values of quantitative variables among the groups. The minimal level of significance was identified at p < 0.05. Correlation coefficient was determined by linear regression analysis 39. Significance of non-parametric data used for analyzing the macroscopic tests (score of bloody diarrhea and lesion

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Table 1. Effects of same doses of Sulfasalazine (SSZ), Dunaliella (DS) and Spirulina (SP) on colon lesion parameters from rats with acetic-acid-induced ulcerative colitis.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Acetic acid</th>
<th>SSZ</th>
<th>DS</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weight</td>
<td>258.5 ± 3.67</td>
<td>238 ± 4.14a</td>
<td>252.125 ± 3.85</td>
<td>263.43 ± 2.91b</td>
<td>271.29 ± 4.51b,c</td>
</tr>
<tr>
<td>Body weight change</td>
<td>32.25 ± 1.95</td>
<td>12.375 ± 0.595a</td>
<td>25.88 ± 2.07b</td>
<td>37.29 ± 1.6b,c</td>
<td>44.71 ± 1.67b,c,d</td>
</tr>
<tr>
<td>Bloody diarrhea</td>
<td>0 ± 0</td>
<td>100 ± 0</td>
<td>12.5 ± 12.5b</td>
<td>57.14 ± 18.89a</td>
<td>14.29 ± 13.36b</td>
</tr>
<tr>
<td>Colon weight</td>
<td>157.0 ± 0.24</td>
<td>2.42 ± 0.028a</td>
<td>1.56 ± 0.03b</td>
<td>1.76 ± 0.02b,c</td>
<td>1.59 ± 0.03b,d</td>
</tr>
<tr>
<td>Colon length</td>
<td>17.34 ± 0.54</td>
<td>12.88 ± 0.305a</td>
<td>15.85 ± 0.35b</td>
<td>14.92 ± 0.47b,c</td>
<td>16.54 ± 0.29b</td>
</tr>
<tr>
<td>Colon weight/length</td>
<td>90.83 ± 2.44</td>
<td>188.61 ± 3.86</td>
<td>98.91 ± 3.24b</td>
<td>119.04 ± 5.37b,c</td>
<td>96.49 ± 2.71b</td>
</tr>
<tr>
<td>Lesion score</td>
<td>0 ± 0</td>
<td>4.88 ± 0.125a</td>
<td>1.38 ± 0.18b</td>
<td>1.71 ± 0.17b</td>
<td>0.57 ± 0.19b,c,d</td>
</tr>
</tbody>
</table>

*Statistical significance as compared to the control.
Statistical significance as compared to the acetic acid-treated group.
Statistical significance as compared to the sulfasalazine-treated group.
Statistical significance as compared to the Dunaliella-treated group.
Table 2. Effects of same doses of Sulfasalazine (SSZ), Dunaliella (DS) and Spirulina (SP) on colon oxidative stress marker and antioxidant parameters from rats with acetic-acid-induced ulcerative colitis.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Acetic acid</th>
<th>SSZ</th>
<th>DS</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/g)</td>
<td>11.89 ± 0.78</td>
<td>56.55 ± 1.87a</td>
<td>32.76 ± 1.84ab</td>
<td>34.94 ± 2.23ab</td>
<td>26.55 ± 1.58ab,b,d</td>
</tr>
<tr>
<td>PCO (nmol/mg protein)</td>
<td>3.79 ± 0.24</td>
<td>8.44 ± 0.28a</td>
<td>4.24 ± 0.14b</td>
<td>6.04 ± 0.24b,c</td>
<td>4.09 ± 0.17b,d</td>
</tr>
<tr>
<td>GSH (mg/g)</td>
<td>1330.25 ± 60.15</td>
<td>645.88 ± 36.4a</td>
<td>859.63 ± 45.87ab</td>
<td>797.43 ± 40.79a</td>
<td>874.57 ± 37.13ab</td>
</tr>
<tr>
<td>CAT (U/g)</td>
<td>24.44 ± 0.73</td>
<td>11.44 ± 0.52a</td>
<td>20.07 ± 1.09ab</td>
<td>15.11 ± 0.95a,c</td>
<td>21.13 ± 1.13b,d</td>
</tr>
<tr>
<td>SOD (U/g)</td>
<td>6.485 ± 0.34</td>
<td>2.845 ± 0.24a</td>
<td>5.2 ± 0.38b</td>
<td>5.71 ± 0.31b</td>
<td>7.67 ± 0.51b,c,d</td>
</tr>
</tbody>
</table>

*aStatistical significance as compared to the control.
*bStatistical significance as compared to the acetic acid-treated group.
*cStatistical significance as compared to the Sulfasalazine-treated group.
*dStatistical significance as compared to the Dunaliella-treated group.

Table 3. Effects of same doses of Sulfasalazine (SSZ), Dunaliella (DS) and Spirulina (SP) on colon inflammation marker and immunomodulatory parameters from rats with acetic-acid-induced ulcerative colitis.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Acetic acid</th>
<th>SSZ</th>
<th>DS</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPO (U/g)</td>
<td>15.88 ± 2.52</td>
<td>59.73 ± 3.19a</td>
<td>37.78 ± 1.74ab</td>
<td>42.64 ± 2.75ab</td>
<td>38.35 ± 1.85ab,b</td>
</tr>
<tr>
<td>TNF-α (pg/g)</td>
<td>61.96 ± 4.44</td>
<td>118.875 ± 5.15a</td>
<td>79.39 ± 3.59b</td>
<td>95.82 ± 5.38b</td>
<td>68.06 ± 3.32bd</td>
</tr>
<tr>
<td>IL-1β (pg/mg tissue)</td>
<td>1.71 ± 0.14</td>
<td>12.21 ± 0.71a</td>
<td>3.805 ± 0.29ab</td>
<td>4.25 ± 0.27ab</td>
<td>3.45 ± 0.30ab,c,b</td>
</tr>
<tr>
<td>IL-6 (pg/mg tissue)</td>
<td>2.055 ± 0.17</td>
<td>14.65 ± 0.85a</td>
<td>4.57 ± 0.36ab</td>
<td>5.105 ± 0.32ab,c</td>
<td>4.145 ± 0.37ab,d</td>
</tr>
<tr>
<td>PGE2 (pg/mg tissue)</td>
<td>202.125 ± 14.3</td>
<td>1535.38 ± 63.19a</td>
<td>712.75 ± 30.47ab</td>
<td>943.29 ± 38.56ab,c</td>
<td>720.14 ± 32.24ab,c,d</td>
</tr>
</tbody>
</table>

*aStatistical significance as compared to the control.
*bStatistical significance as compared to the acetic acid-treated group.
*cStatistical significance as compared to the Sulfasalazine-treated group.
*dStatistical significance as compared to the Dunaliella-treated group.

Table 4. Scoring the severity of the histopathological alterations in colon of different experimental groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Acetic acid</th>
<th>SSZ</th>
<th>DS</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucosal ulceration</td>
<td>--</td>
<td>++</td>
<td>--</td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>Mucosal necrosis</td>
<td>--</td>
<td>++</td>
<td>--</td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>Mucosal hemorrhage</td>
<td>--</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>--</td>
</tr>
<tr>
<td>Submucosal edema</td>
<td>--</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Submucosal hemorrhage</td>
<td>--</td>
<td>++</td>
<td>--</td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>Submucosal inflammatory cells infiltration</td>
<td>--</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Submucosal congestion</td>
<td>--</td>
<td>+</td>
<td>+</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

+++: Severe histopathological alteration.
++: Moderate histopathological alteration.
+: Mild histopathological alteration.
--: Nil histopathological alteration.

score) was achieved using GraphPad Prism software version 5 (Graph Pad Software Inc., San Diego, CA) and was evaluated by the Kruskal–Wallis test [non-parametric ANOVA] followed by Dunn’s multiple comparisons test, p < 0.05.

Results

Macroscopic scoring and colon lesion parameters

The body weight and macroscopic lesion parameters are presented in Table 1. Acetic acid caused severe macroscopic edematous inflammation in the colon, as assessed by the high score of colonic lesion and bloody diarrhea, in addition to the increased colon wet weight and colon weight/length. *Spirulina platensis* and *Dunaliella salina* significantly ameliorated all these lesions, and both were comparable to the standard drug; SSZ effects except DS which failed to affect the bloody diarrhea score.

Oxidative stress markers

The overwhelmed defense systems resulted in a significant increase in concentration of MDA and PCO by 476 and 223% in acetic acid ulcerated group compared to the control group (Table 2). Regarding the redox state, acetic acid group caused a significant decrease in colonic non-enzymatic; GSH by 49% and enzymatic; CAT, SOD defense systems by 47 and 44% respectively; however, these effects were significantly reversed in all treated animals of groups 3, 4 and 5, approaching the normal values with SP treatment. Treatment with SSZ, DS or SP produced a marked decrease in MDA by 58, 62 and 47% and in PCO levels by 50, 72 and
48% respectively, compared to the acetic acid ulcerated group. Moreover, the levels of GSH were increased by 133, 123.5 and 135% in SSZ-, DS- and SP-treated groups. Likewise, the levels of CAT and SOD were also elevated by 175 and 483% in SSZ group, by 132 and 201% in the DS-treated group and finally by 185 and 269% in the SP-treated group.

**Inflammatory and immunomodulatory markers**

In acetic acid ulcerated rats, the macroscopic inflammatory effect was mirrored by a 376% increase in mucosal MPO activity, compared to the control group. SSZ, DS and SP produced a significant reduction in MPO activity compared to the acetic acid ulcerated group by about 63, 71 and 64%, respectively (Table 3). Ulcerated non-treated group showed an elevation by 192% in the colonic TNF-α level, which was reversed upon treatment with SSZ, DS and SP by 67, 81 and 57%, respectively. Likewise, IL-1β level increased by 714% in the acetic acid group compared to the control group, and decreased by about 31, 35 and 28% in SSZ, DS and SP treatment groups. Moreover, IL-6 and PGE₂ levels were increased in the acetic acid non-treated group by 713 and 759% compared to the control group. Although this effect was lowered by all treatment regimens, yet the effect of SSZ and SP 500 mg/kg was more pronounced than DS-treated groups. The reduction levels in IL-6 level were 31, 35 and 28% for SSZ, DS and SP groups, respectively. Similarly, the PGE₂ reductions level for these groups was 46, 61 and 47%, respectively.

**Histopathological studies**

The previous macroscopic findings were emphasized by the histopathological examination, where the severity of alterations in the colon of different experimental groups was microscopically scored blindly by an independent
histopathologist, and the scores are presented in Table 4. In the colon of the negative control group, there was no histopathological alteration and the normal histological architecture was recorded (Figures 1A, 2A, 3A, and 4A).

The microscopic pattern of acetic acid-induced colitis group is characterized by focal ulceration, necrosis and hemorrhage were noticed in the mucosal layer associated with hemorrhage, edema and inflammatory cells’ infiltration in the submucosa (Figure 1B). The intact regenerative crypts were lined with columnar absorptive and goblet cells with marked mucin depletion (Figures 2B and 3B). In addition to a significant increase in the lymphocytic infiltration, extravasated red blood cells and different types of leukocytes were noticed among the colonic tunics. The cellular infiltration is more extensive towards the upper mucosal surface between the crypts and extends diffusely towards the submucosa and even towards the muscularis externa (Figure 1B). The stromal elements separating the mucosal crypts were rich in thickened collagenic layer, and a lesser extent of collagenic fibers were noticed toward the colonic submucosa (Figure 4B). The SSZ-treated group was significantly improved as compared to the experimental colitis group, where the mucosal layer showed focal hemorrhagic areas while the underlying submucosa showed edema, few inflammatory cells’ infiltration and dilated blood capillaries (Figure 1C). The columnar absorptive cells as well as the goblet cells comprised fewer amounts of neutral and acidic mucin as compared to the normal control group. Fine collagen fibers and engorged blood vessels were noticed among the colonic lamina propria and submucosa (Figures 2C, 3C and 4C).

Figure 2. Photomicrographs of colonic sections of different treatment groups stained by PAS. (A) Control group (vehicle treated) showing strong PAS reaction in the absorptive columnar and goblet cells. (B) Acetic acid-treated group (AA) showing marked neutral mucins depletion. (C) Sulfasalazine-treated group (SSZ) showing strong PAS positive columnar and goblet cells. (D) Dunaliella-treated group (DS) showing strong PAS positive goblet cells. (E) Spirulina-treated group (SP) showing moderate PAS positive reaction in the goblet cells.
Moreover, histological findings in the DS-treated group showed focal ulceration with necrosis and hemorrhages in the mucosal layer while the underlying submucosa showed hemorrhages, edema and inflammatory cells infiltration (Figure 1D). The columnar absorptive cells as well as the goblet cells showed more neutral and acidic mucins content as compared to the SP-treated group (Figures 2D and 3D). Both lamina propria and submucosa comprised nearly normal content of collagen fibers as compared to the control group (Figure 4D). On the other hand, histological examination revealed that the group treated with SP showed a substantial reduction of the mucosal epithelial erosions and marked decreasing of the cellular infiltrate as compared to the experimental group. Furthermore, a slight variation in crypt architecture was also marked, as they are often regular in shape and size (Figure 1E). Marked increase in neutral and acidic mucin contents of goblet cells were also observed, particularly alcianophilic goblet cells (Figures 2E and 3E).

Masson’s trichrome stained colonic sections showed fine collagen fibrous content of the lamina propria as compared to the control group (Figure 4E).

**Immunohistochemical studies**

The underlying inflammatory mechanisms were then investigated by immunohistochemical evaluation of iNOS and the PG-generating enzyme, COX-2 expression. Immunoreactivity of the COX-2 protein was generally intense than that of iNOS in the different treatment groups. However, the area
percentage of immunoreaction in different treatment groups for COX-2 expression was strongly correlated with that of iNOS expression ($r = 0.865$, $p < 0.0001$). No immunoreaction was detected in the control group for either COX-2 or iNOS (Figures 5A and 6A). On the other hand, severe positive immunohistochemical signal for COX-2 and iNOS was detected in ulcerated non-treated samples (Figures 5B and 6B). Positive cells typically exhibited a brown reaction product without background staining. The immunoreaction area percentage varied among histologic structures in different treatment groups, where the immunoreaction in the SSZ-treated group was mild for COX-2 and moderate for iNOS (Figures 5C and 6C). Sections from DS-treated rats showed moderate immunohistochemical signals for both COX-2, and iNOS (Figures 5D and 6D). Conversely, sections from SP-treated rats showed no and mild immunohistochemical signal for COX-2 and iNOS, respectively (Figures 5E and 6E). The mean areas of immunohistochemistry for the control, acetic acid, SSZ-, DS- and SP-treated samples are $2.06 + 0.62$, $27.25 + 1.74$, $8.95 + 1.93$, $16.27 + 1.75$ and $6.74 + 0.62$ for COX-2 (Figure 5F), and $1.1 + 0.28$, $33.4 + 2.28$, $12.035 + 1.88$, $19.01 + 1.4$ and $4.18 + 0.85$ for iNOS (Figure 6F), respectively.

**Correlation studies**

Estimation of colonic damage by mucosal injury scoring was found to be strongly correlated with the mucosal content of MDA, PCO, GSH, CAT and SOD ($r = 0.89$, $0.88$, $-0.7$, $-0.79$ and $-0.77$, respectively, $p < 0.0001$) as shown in...
Figure 7(A–E). Estimation of colonic damage by mucosal injury scoring was found to be strongly correlated with the mucosal content of MPO, TNF-α, IL-1β, IL-6 and PGE2 ($r = 0.8, 0.81, 0.935, 0.935$ and $0.89$ respectively, $p < 0.0001$) as shown in Figure 8(A–E).

Discussion

Ulcerative colitis is an IBD characterized by inflammation of the colorectal mucosa. Dysregulation in the immune response, with infiltration of leukocytes into the mucosal interstitium play an important role in its pathogenesis together with excessive production of ROS$^2$. The effects of acetic acid on the colon in our UC experimental model were explained by the fact that acetic acid could trigger inflammation by many biological pathways, including direct cytotoxic effects in addition to apoptotic damage of colonic epithelial cells$^{25}$. In addition, there is an increase in colonic weight/length confirms intensification of intestinal infiltrations, inflammation and consequent intestinal edema$^{40}$. The present investigation outlines the anti-inflammatory and antioxidant activities of DS and SP against acetic acid-induced ulcerative colitis. Treatment of rats with DS or SP significantly reduced the wet weight of distal colon segments, the gross lesion scores, the incidence of diarrhea and occult blood and significantly inhibited colonic shortening. However, regarding the final body weight change, SP and DS offered better safety profile, compared to the acetic acid-treated groups.

Oxidative stress plays a fundamental role in disease initiation and progression of IBD$^{41}$. Furthermore, it is well known that the infiltration of inflammatory cells is considered as a trigger of free radical production, which then attack cellular macromolecules, disrupt epithelial cell integrity, perturbate membrane lipids and delay mucosal recovery through the impairment of endogenous defense systems$^{42}$. In this study, acetic acid-induced ROS formation, as indicated by
elevation of PCO levels, and reduction of SOD and CAT activities. Moreover, acetic acid-induced oxidative stress and lipid peroxidation was indicated by the increase in MDA, and decrease in GSH levels. Similar findings have been previously reported by Mustafa et al. On the contrary, PCO and MDA levels were reduced while SOD, CAT activities and GSH levels were elevated in all treated groups, especially SP group, which even showed better improvements than SSZ.

In addition, measurement of MPO activity has been previously regarded as an indicator of the neutrophil influx into inflamed gastrointestinal tissue. A previous study reported that acetic acid increased the levels of colonic MPO, indicating infiltration of neutrophils and perturbation of the inflammatory system. This result is observed in mice models, rat models, as well as patients with IBD. In agreement with previous reports, the present study showed a significant increase in MPO activity in the acetic acid group. On the other hand, both SP and DS 500 mg/kg ameliorated neutrophil infiltration as evidenced by suppression of colon MPO.

Furthermore, macrophages produce TNF-α and IL-1β, the levels of which are often elevated in both animal models and patients with UC. IL-1β and TNF-α, are often regarded as key immunoregulatory cytokines that amplify the inflammatory reaction by triggering a cascade of immune cells, hence stimulating production of cytokines, arachidonic acid metabolites, and proteases by intestinal macrophages, neutrophils, smooth muscle cells, fibroblast and epithelial cells. In addition, IL-1β and TNF-α induce epithelial cell necrosis, edema, neutrophil infiltration, stimulate proliferation of intestinal smooth muscle cells and fibroblasts and induce synthesis of IL-6, IL-8 and PGE2 by these cells. In this context, it had been documented that blocking of the action of endogenous IL-1β and TNF-α attenuates acute and chronic experimental colitis and its further systemic complications.

Moreover, it is well documented that during the pathologic course of experimental colitis, TNF-α and IL-1β are released.
with subsequent activated synthesis of PGE2 and exacerbation of tissue damage. In this study, acetic acid caused elevations in colonic levels of TNF-α and PGE2, which could mediate epithelial cell necrosis, edema and neutrophil infiltration. These elevations are in harmony with the previous finding that TNF-α is widely expressed in the gut of IBD patients. Moreover, augmented levels of PGE2 goes in harmony with previous studies which proved that elevated level of PGE2 is explained by its enhanced synthesis rather than reduced catabolism, both of which are mediated by TNF-α.

Importantly, SP and DS decreased significantly the production of both TNF-α and PGE2 compared to the acetic acid group. Finally, the antiulcerogenic morphological effect of DS or SP was further confirmed by histological preservation of the colon architecture and correlated to its anti-inflammatory properties. Treatment with either SP or DS significantly attenuated the extent and severity of the histological features of cell damage compared to the acetic acid group, an effect which was less observed in the DS-treated group. In the present study, immunohistochemistry showed significantly increased COX-2 expression in the ulcerative lesions, suggesting that endogenous PGs production may be involved in exacerbation of the underlying ulcerative damage. This concept is in agreement with a previous study, as higher level of PGE2 was detected in the ulcerated group than in the treated groups. Furthermore, in the current study, immunoreactivity of COX-2 was correlated to that of iNOS. Our results are in agreement with several previous observations.

Figure 7. Correlation analysis. Analysis of the correlation coefficients between gross lesion score and MDA (A), PCO (B), GSH (C), CAT (D) and SOD (E). A significant positive correlation was observed between gross lesion score and colonic MDA, PCO, GSH, CAT and SOD ($r = 0.89, 0.88, 0.7, 0.79$ and $0.77$ respectively, $p < 0.0001$).
that suggest an interaction between COX-2 and iNOS\textsuperscript{51,52}. Inflammatory cells, such as neutrophils and macrophages, express iNOS, produce NO, and also produce superoxide\textsuperscript{53}. Thus, iNOS is considered as a hallmark of inflammation, which is consistent with previously published data documenting elevated mRNA levels of iNOS in the colon of ulcerated rats\textsuperscript{54}. The iNOS upregulation during inflammation is not surprising since iNOS has been reported to be localized in infiltrated neutrophils and macrophages in the colonic mucosa and submucosa in animal models of IBD\textsuperscript{55}. 

To our knowledge, the antioxidant and anti-inflammatory effects of SP or DS have not been examined previously in the models of UC. In accordance with our results, it was proven that the antioxidant properties of SP\textsuperscript{24} and DS\textsuperscript{16} contributed to its beneficial effect in treating various pathological conditions.

**Conclusion**

This study suggests potential benefits of SP and DS in an experimental model of colitis at the tested dose (500 mg/kg). These effects, which are comparable or even better than SSZ in case of SP, and are possibly attributed to their anti-inflammatory and antioxidant properties. Hence, a strategy that addresses the possible therapeutic efficacy of SP and DS in the treatment of UC, following the appearance of its symptoms, should merit further investigations.

**Declaration of interest**

All authors declare that they have no competing financial or personal interest or any kind of conflict of interest relevant to this study.
References


