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Interventional Effects of Plumbagin on Experimental Ulcerative Colitis in Mice

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Abstract

Plumbagin (**1**) is a naphthoquinone constituent of plants that have been used in traditional systems of medicine since ancient times. In the present study, the role of **1** was examined on the amelioration of ulcerative colitis, an inflammatory bowel disease that is not curable currently. Plumbagin was tested at a dose of 6-10 mg/kg body weight in acute and chronic disease models. Diseased mice receiving **1** at 8-10 mg/kg demonstrated a significant suppression of disease symptoms in both models. However, body weight loss was not restored in either of the models. Levels of proinflammatory cytokines (TNF-α, IFN-γ, and IL-17) were reduced significantly by **1** in mice suffering from chronic disease, while cytokine levels remained unaffected in mice with acute disease. However, the percentage of inflammatory (CD14+/CD16+) monocytes present in peripheral blood was significantly reduced (>three-fold) (p <0.05) in treatment groups relative to controls in the acute model. Histological evaluations exhibited the restoration of goblet cells, crypts, and the submucosa along with a significant reduction in monocyte aggregation in colon sections from mice receiving treatment with **1**. Restoration in colon size was also observed in the treatment groups.

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone, **1**) is a naturally occurring yellow pigment derived from the medicinal plant, *Plumbago zeylanica* L. (Plumbaginaceae). The root of *P. zeylanica* (“chitrak”) has been used in Indian traditional medicine (Ayurveda) since at least 750 BCE, as an antiatherogenic, cardiotonic, hepatoprotective, and neuroprotective agent.[1] Plumbagin is also derived from black walnut and some other plants.[2] Recently, **1** was shown to exert antimicrobial and antiproliferative activities in cell culture and antitumor activity in vivo.[1-4] Ulcerative colitis (UC) is a chronic inflammatory condition in the human gastrointestinal tract, affecting mainly the colon and rectum. This is an intermittent disease, with periods of exacerbated symptoms and those that are relatively symptom-free. Problems associated with UC include bloody diarrhea, colon ulcers, weight loss, long-term and increased risk of colon cancer and unknown pathologies.[5] UC primarily affects the mucosal lining of the colon and rectum.[5] Polymorphonuclear neutrophil infiltration is regarded as the primary and central lesion of colitis that is usually followed by loss of the epithelium, loss of goblet cells, and crypt damage. Five percent of all people diagnosed with UC are eventually diagnosed with colon cancer.[6] Ulcerative colitis may occur in persons of any age, but most often it starts between ages of 15 and 30, or less frequently between ages of 50 and 70.[7] American Jews of European descent are four to five times more likely to develop UC than the general population. UC is diagnosed predominantly in developed countries.[8]

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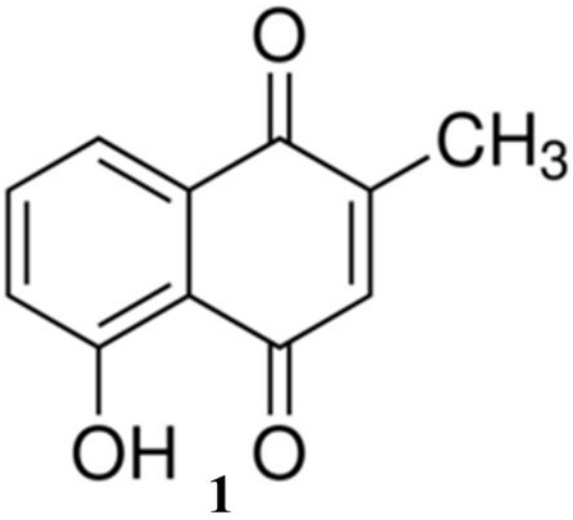
The pathogenesis of ulcerative colitis is not well-known. However, it is generally agreed that UC is a multi-factorial disease with genetic, environmental, and immunological factors playing roles in its development. The major theories include prior infection, allergy to food components, genetics, environmental factors, and an immune response to bacteria or other antigens.[9] In addition, the results of genetic studies and mouse models have emphasized the role of genetic predisposition and how this affects interactions with microbial and environmental factors, leading to pro-colitogenic perturbations of the host–commensal relationship.[10] There is no known cure for UC. Various treatment options include anti-inflammatory, immunosuppressive, and antibiotic drugs. However, these treatments lead to limited remission, have significant side effects, and yield refractory patients.[11] Thus, there is an urgent need for effective, alternative therapies.

Plumbagin (**1**) has gained much interest recently because of its potential to affect major inflammatory pathways. It was reported to suppress nuclear factor-$_{\kappa}$B (NF-κB) activation and NF-κB-regulated gene products through modulation of p65 and IκBα kinase activation, leading to potentiation of apoptosis in cancer cells.[2,12] Alhough the precise mechanisms involved in the immunopathology of ulcerative colitis are not known, the upregulation of the NF-κB pathway has been well-evidenced.[5] An anti-inflammatory and analgesic effect of **1** through the inhibition of NF-κB activation was also reported by Luo et al.[12] Another study reported its role in the inhibition of the STAT3 activation pathway that is critical to the activation of genes preceding chronic conditions including ulcerative colitis.[13] Therefore, the role of **1** in a murine disease model of colitis was examined in this study. There is no published report on the effect of **1** on inflammatory bowel disease. It was hypothesized that **1** would reduce inflammatory responses and cause remission in disease symptoms in mice by downregulating the NF-κB or STAT3 pathways. Thus, the objectives of this research were to induce chemically UC in C57-BL6/J mice and to study the effects of treatment with **1** on (i) clinical markers, (ii) histopathology of the mouse colon, and (iii) inflammatory cytokines such as TNF-α, IFN-γ, and IL-17 in acute and chronic models.



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Results and Discussion

Dextran sodium sulfate (DSS)-induced colitis is one of the most common experimental models used to study the pathogenesis of inflammatory bowel diseases.[14] Figure 1 displays the acute disease activity index for different experimental groups. A validated index (1 indicating no disease; 4 indicating the most severe disease) of average scores based on the combination of clinical symptoms (body weight, diarrhea, fecal occult blood/prolapse and

colon length) was constructed (Table 1). It can be seen that the vehicle control representing healthy mice had a starting value of 1, while the DSS-disease control had a value of 4 (Figure 1). In the treatment groups, the disease index improved with increasing concentrations of plumbagin (**1**). The groups receiving 8 mg/kg body weight (PL-8) and 10 mg/kg body weight (PL-10) of **1** demonstrated a significant suppression of clinical symptoms of ulcerative colitis in this model. The effect of **1** on the disease index was comparable to improvements achieved with dietary phenethyl isothiocyanate.[6] However, body weight restoration in the treatment groups was not significantly different ($p < 0.05$) from the DSS-disease controls (data not shown). DSS-induced ulcerative colitis is characterized by diarrhea, anal bleeding, and weight loss. The treatment thus aims at controlling the weight loss in addition to improvement in other clinical symptoms, but treatment with **1** was not observed to reverse weight loss. This may have occurred due to the severe tissue damage caused by DSS at 3% (w/v). In addition, the dose of **1** used in the present experiment may have been suboptimal, as one report indicated the efficacy of a higher dose of plumbagin (20 mg/kg body weight) in the inhibition of NF-κB-activated paw edema of rats.[12] Pharmacokinetics of plumbagin in rats indicated the oral bioavailability of plumbagin to be nearly 38% only.[4] The reversal of DSS-induced weight loss by the action of extracts of *Ginkgo biloba* L. (Ginkgoaceae) and *Panax quinquefolius* L. (Araliaceae) have been reported earlier.[15,16] However, one of the major differences between prior studies and this investigation was the use of DSS at 1% (w/v). It is likely that the lower dose of DSS used in the cited studies resulted in less severe damage to the colonic epithelium as compared to the 3% of DSS used in the present study. Further, a recent investigation indicated that the severity of colitis can differ between similar DSS preparations of the same molecular weight range.[14] This difference in colitogenic properties may be affected by the total sulfur content of each DSS preparation. The DSS used in the present investigation was reported to cause the most severe disease and weight loss.[14]

Several mechanisms for DSS-induced colitis have been proposed in the literature. The rapidity of the acute model suggests that adaptive immunity is not pivotal to the development of lesions or crypt erosion in the colonic mucosa.[14] Furthermore, no precise inflammatory mechanisms have been identified that contribute to the genesis of acute lesions. Similar DSS-induced lesions were also observed in the case of severe combined immunodeficient mice, thus ruling out any involvement of B- or T-cells in the pathology.[14] The resultant colonic inflammation is driven predominantly by innate cell types. However, some recent reports also indicate the involvement of CD4+ T-cells in the acute model induced by DSS.[11,15] In this context, the pathogenic mechanism responsible for the acute colitis can be attributed to the direct toxic effects of DSS on colonic epithelial cells. Bomba et al.[14] demonstrated convincingly apoptotic properties of DSS on epithelial cells correlating with the sulfur content in each DSS preparation.

It was shown recently that the genotoxic damage in colitis extends beyond the site of inflammation to circulating leukocytes and erythroblasts in the bone marrow, manifesting a systemic effect.[17] Therefore, circulating monocytes were phenotyped as classic (CD14+/CD16-) or inflammatory (CD14+/CD16+) using flow cytometery in this study (Figures 2A, 2B). The percentage of circulating inflammatory monocytes in DSS-disease control mice was significantly different ($p < 0.05$) from the levels of inflammatory monocytes in the treatment groups and in the vehicle control group (Figure 2A, 2B). The percentage of inflammatory monocytes was significantly less (>three-fold) ($p < 0.05$) in the treatment groups, particularly in PL-8 and PL-10 groups, as compared to the DSS disease group. Also, these values were not significantly different ($p < 0.05$) than the vehicle control. Inflammatory monocytes are recruited rapidly to sites of inflammation, but their excessive and/or prolonged recruitment hinders the resolution of inflammation and is a hallmark of numerous diseases including ulcerative colitis.[18] It is possible that **1** interferes with monocyte

recruitment reducing total counts during the treatment period, resulting in a significant reduction in the disease index in the PL-8 and PL-10 treated mice. Monocytes play a pivotal role in the inflammatory cascade and are a major source of both pro-and anti-inflammatory cytokines. They are involved intimately in tissue damage and repair and an imbalance of these processes may have detrimental consequences, as in UC.[18] However, it was surprising to observe that the proinflammatory cytokines TNF-α, IFN-γ, and IL-17 in the treatment groups were not significantly different than in the DSS-disease control, when cytokines were quantified from acute disease sera (data not shown). The cytokine responses characterizing the inflammatory bowel diseases are the key pathophysiologic elements that govern the initiation, evolution, and, ultimately, the resolution of these forms of inflammation.[19]

Disease activity indices built on the disease scores from the chronic disease model revealed suppression effects of plumbagin (**1**) in the PL-8 and PL-10 treatment groups, similar to those of the acute model (data not shown). However, the activity of proinflammatory cytokines in this disease model exhibited a different pattern, as seen in Figures 3-5. It is evident from Figures 3 and 4 that levels of TNF-α and IFN-γ were decreased significantly ($p < 0.05$) in the treatment groups, particularly for the PL-10 group, when compared to the DSS-disease control. Mice in the vehicle control group showed no detectable levels of TNF-α. The dramatic decrease in TNF-α concentration in the treatment groups suggests a positive effect of intervention by **1** in this model (Figure 3). TNF-α plays a key role in colitis.[19] It is interesting to observe that the level of IFN-γ was even less ($p < 0.01$) than in the vehicle control group (Figure 4). The plumbagin-mediated reduction in serum levels of TNF-α and IFN-γ observed in the current study was comparable to that reported previously for resveratrol-treated mice in the disease model of colitis.[11] The cytokine response was also consistent with an earlier report of an anti-inflammatory effect of **1** in a rat model of paw edema.[12] Proinflammatory cytokines such as TNF-α, IL-1β, IFN-γ, and IL-6 are generally overproduced during inflammatory bowel disease. Blocking IL-1 or TNF has been highly successful in patients with rheumatoid arthritis, inflammatory bowel disease, or graft-vs.-host disease.[19]Figure 5 shows the concentration of IL-17 in sera from mice in the chronic disease model. The pattern of IL-17 activity in the treatment groups was far from clear. As seen in Figure 5, there were significant differences ($p < 0.05$) in IL-17 levels between the PL-8 and PL-10 groups. PL-8 mice exhibited significantly elevated levels ($p < 0.05$) of IL-17 as compared with the DSS control group. In turn, PL-10 mice had significantly lower levels ($p < 0.05$) of IL-17 relative to the DSS-disease control group. The role of IL-17 in colitis is yet unclear and conflicting results have been reported.[19,20] It has been shown that IL-17F deficiency results in reduced colitis, indicating that IL-17F has a pathogenic role in colitis. IL-17A null-mice develop more severe disease, suggesting a protective role. It appears that Th17 lymphocytes secrete IL-17F or IL-17A/IL-17 based on the mucosal internal milieu.[20] This may explain the conflicting effects of different doses of **1** in the present study. More recently, studies have been published showing that IL-17 levels were increased in mice with UC compared to controls, but this increase was found to be far less than observed in Crohn's disease.[19]

Random histological samples of the colorectal region from both the DSS-disease control and treatment groups were examined. In the disease control mice, heavy disruption of crypts and goblet cells accompanied by monocyte aggregation in the mucosal region were observed (Figure 6). It can be seen that goblet cells and crypts were no longer intact and these areas were heavily invaded by lymphocytes. Plumbagin treatment resulted in a restoration of goblet cells, crypts, and submucosa along with a significant reduction in monocyte aggregation (Figure 7). Similar changes in UC histopathology were observed when diseased mice were treated with resveratrol and dietary phenethyl isothiocynate.[6,11] The treatment effects were also evident in the size of the colorectal region. Figure 6 indicates that the colon size in treatment groups, similar to the vehicle control, was significantly different than in

DSS-disease group. DSS is known to induce shrinkage of the colon due to inflammation, constriction, and ulceration. These results suggest that **1** restores the size of the colon, affecting the histology of colorectal regions.

In summary, plumbagin (**1**) interferes with the physiopathogenesis of colitis reducing disease severity, although not completely reversing the disease process. The effects on clinical symptoms, histology, and size of the colon were significant. Elevated levels of circulating inflammatory monocytes ((CD14+/CD16+) and cytokines (TNF-α and IFN-γ) were reduced significantly with plumbagin intervention, although body weight loss was not restored in any of the models used. One of the possible reasons for the limited effects observed could be ascribed to the possible non-optimal dosage of **1** used in this investigation. Further investigations will be needed to confirm the efficacy of this natural product and to determine optimal doses for maximum benefit in this murine model of ulcerative colitis.

Experimental Section

Induction of Ulcerative Colitis by DSS Administration – Acute Model

Various groups of 8-10 week old female mice (C57-BL6/J), each of five replicates, were housed in a standard mouse cage with a common supply of chow and drinking water. All groups were given the same chow (Lab Diet, PMI Nutrition International, LLC, Cincinnati, OH, USA) ad libitum. Dextran sodium sulfate (DSS; 36,000--50,000 Da) obtained from MP Biomedicals (Santa Ana, CA, USA) was administered in the following sequence.[21] Before the start of DSS treatment, each group of mice was weighed and marked. The drinking supply of the mouse cages was filled with 3% (w/v) DSS solution in the DSS-disease control group and in the three treatment groups. Consumption of 5 mL DSS solution per mouse per day was projected. Vehicle control mice received the same drinking water without DSS. The remaining DSS solution from water bottles was emptied at day 3 and refilled with DSS solution for another two days. The remaining DSS solution from the bottles was emptied at day 5 and refilled with DSS solution. The remaining DSS solution was replaced by autoclaved water on day 8 for all groups.[21] Mice were weighed at each transfer. The study protocol was approved by the Institutional Animal Care and Use Committee of Western Kentucky University (Approval# 09-04).

Plumbagin (1) Treatment – Acute Model

Plumbagin (**1**), with >97% purity (Sigma-Aldrich, St. Louis, MO, USA), was dissolved in ethanol before its use at different doses. A group of mice receiving ethanol (270 μL/100 mL water) in place of DSS was maintained as vehicle control. Mice in a second group were exposed to DSS alone and were maintained as a DSS-disease control group. Other groups exposed to DSS were treated with **1** after the induction of disease (starting at day 6) in the amount of 6, 8, and 10 mg/kg mouse body weight, respectively. The calculated volume of **1** was mixed with 100 mL of drinking water per bottle/cage for mice consumption ad libitum. The treatment continued until termination of the experiment.

Monocyte Phenotyping

Monocytes were phenotyped as classic (CD14+/CD16-) or inflammatory (CD14+/CD16+), according to a modified published protocol.[22] All antibodies and buffers were obtained from eBioscience (San Diego, CA, USA). Briefly, whole blood from each replicate of different groups was obtained and 20 μL were added to a 250 μL antibody panel containing titered quantities of anti-mouse CD14-FITC and anti-mouse CD16-PE in a flow cytometry staining buffer. The sample was incubated for 30 min in the dark and then centrifuged for 5 min. After decanting and vortexing thoroughly, 300 μL of RBC lysis buffer were added and the

sample was incubated for 15 min before 300 μL of PBS were introduced. The sample was centrifuged for 10 min, decanted, and then vortexed thoroughly prior to analysis by flow cytometry (BD Accuri, C6, San Jose, CA, USA). An initial monocyte gate was established according to forward- and side-scatter properties, and then populations of classic and inflammatory monocytes were identified (Figure 2B). Each replicate sample was processed in duplicate and averaged to obtain the final result.

Induction of Ulcerative Colitis and Plumbagin Treatment (1) – Chronic Model

The published protocol of disease induction developed by Wirtz et al.[21] was modified with intervention by **1** as follows. The duration of the experiment was 63 days. For 1 week, mice in the DSS-disease control and the treatment groups were exposed to DSS. Following DSS exposure, the DSS-disease control mice were administered water and the treatment groups were administered **1** for 1 week. The DSS-disease control and treatment groups were then supplied with water alone for 1 week. This cycle was repeated two more times to complete the establishment of the chronic model. The vehicle control group was maintained on water (no DSS or 1) throughout the duration of the experiment

Clinical Markers

Body Weight Measurement—The body weight of mice in all experiments was measured in g every other day from day 1 to the experiment termination day. The percent change in body weight was calculated.

Diarrhea and Fecal Blood—For diarrhea, the stool of each group was observed and scored on a scale ranging from 1 to 4 based on its consistency: 1 = normal solid; 2 = easily severed; 3 = easily crushed into paste; and 4 = loose liquid spots, sticking to the cage walls and the mouse body. Levels of fecal occult blood in stool samples were measured at the time of body weight measurements using a Colo Screen kit (ES Lab Pack, Helena Laboratories, Beaumont, TX, USA). Scoring on the fecal occult was completed as depicted in Table 1. The rectum was also examined for prolapse, swelling, or bulging.

Disease Index—An index was prepared on the basis of scores on clinical markers as in Table 1.

Euthanasia and Isolation of Colon—Mice from each group were euthanized using an overdose of the inhalant anesthetic, isofluorane (Baxter Healthcare Corporation, Deerfield, IL, USA), with 99.9% isofluorane/mL administered in an euthanasia chamber. After ensuring death, each mouse was dissected for the isolation of the colorectal region. Isolated colorectal lengths were measured in centimeters. Colorectal samples were stored in 10% neutral formalin solution prior to histological analysis.

Histology

Paraffin-embedded tissues were sectioned at a thickness of 6-8 microns using a microtome. The sectioned ribbons containing colon sections were then stained with hematoxylin and eosin (H-E) for microscopic study.[23] In a typical tissue, nuclei stain blue, whereas the cytoplasm and extracellular matrix gave varying degrees of pink staining.

Enzyme-Linked Immunosorbent Assay (ELISA) for Cytokine Quantification

Blood was drawn from each mouse and stored overnight. Clotted blood samples were then subjected to centrifugation for 10 min at 3,000x*g*. Sera from different groups were used for testing the concentrations of TNF-α, IFN-γ, and IL-17. Anti-mouse TNF-α, IFN-γ, and IL-17 antibodies were procured from R&D Systems, Inc. (Minneapolis, MN, USA), and

solutions and reagents were prepared following the R&D Systems supply kit. A 96-well microplate was coated with 100 μL per well of the diluted capture antibody. The plate was sealed and incubated overnight at room temperature. Then, 100 μL of sample or standard were added in reagent diluent, per well. The microplate was covered with an adhesive strip and incubated for 2 h at room temperature. After washing three times with wash buffer, 100 μL of the detection antibody, diluted in reagent diluent, were added to each well. Next, 100 μL of the working dilution of Streptavidin-HRP were added to each well for 20 min. Substrate solution was added at the rate 100 μL per well. At the end, 50 μL of stop solution were added to each well before measuring the optical density. The optical density of each well was determined immediately, using a microplate reader (Synergy H1 hybrid reader; Bio Tek, Winooski, VT, USA) set to 450 nm.

Statistical Analysis

The data were expressed as means ± standard error of 8-10 replicates for each group. Differences between disease control and treatment groups or vehicle control were analyzed by one-way analysis of variance with Tukey's posthoc tests using SYSTAT (Version-19). A p value of <0.05 was considered as statistically significant in each experiment.

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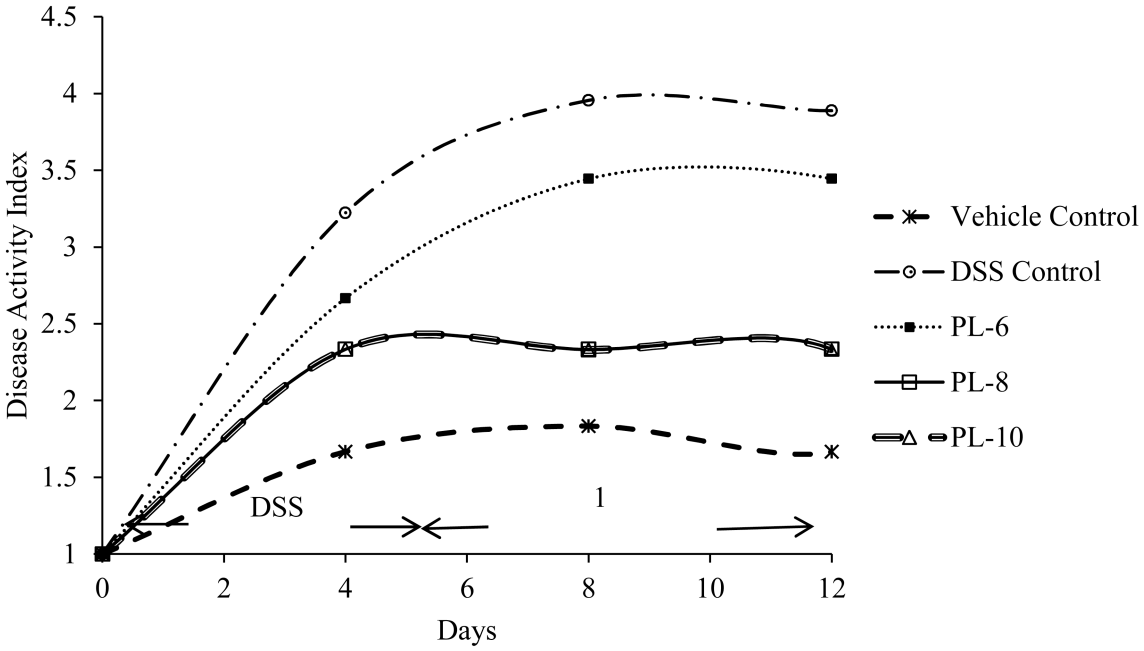

Figure 1.

Effects of plumbagin (**1**) on the disease activity index in an acute model of disease. Disease index scoring criteria are described in Table 1. Values for **1** treatment at 8 and 10 mg/kg (body weight) were significantly different (p <0.01) with respect to DSS-disease control. Data represent the means of two experiments (n = 10) at a four-day interval.

Figure 2A.

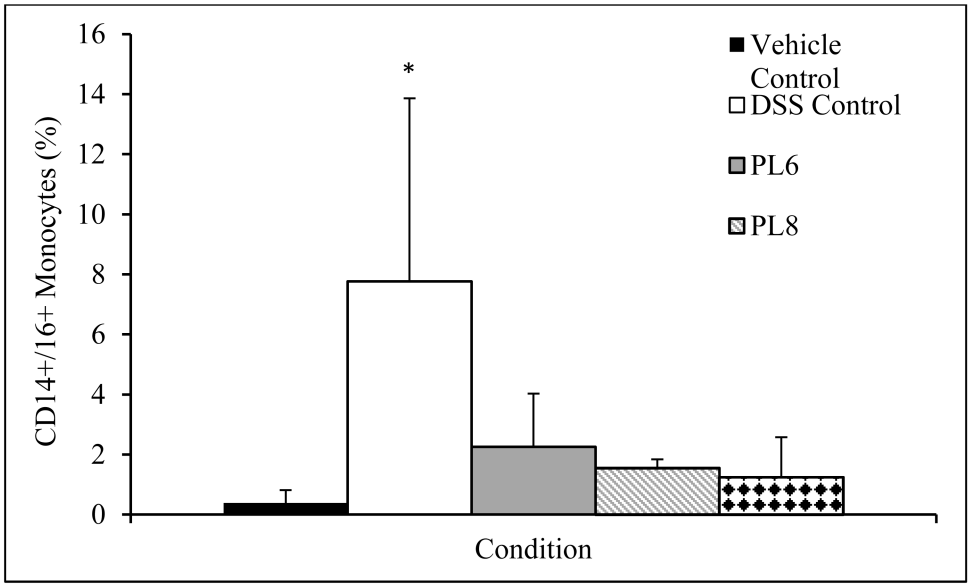

Figure 2B.

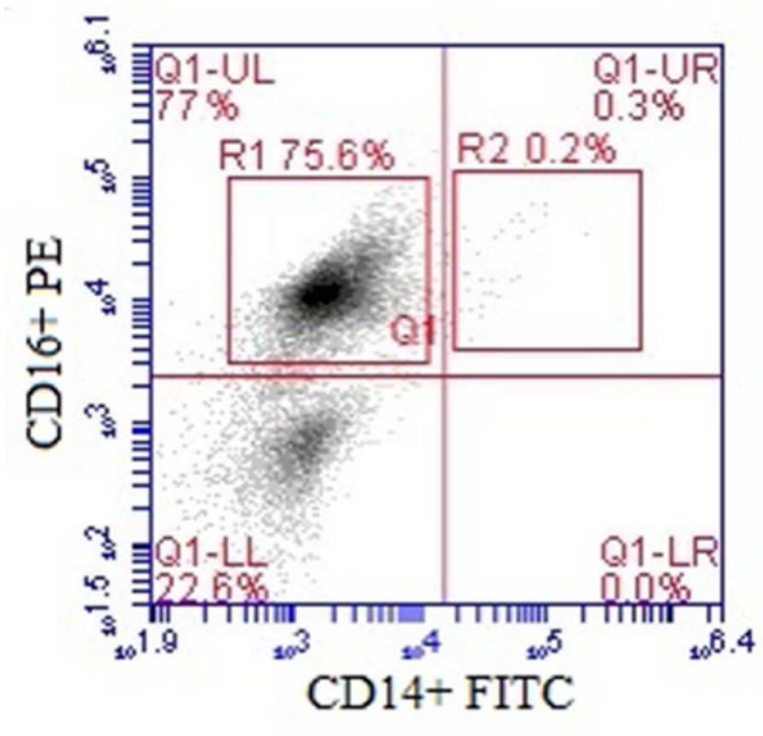

Figure 2.

A. Percentage of inflammatory monocytes in mice in plumbagin (**1**) treatment groups (PL-6, -8, -10 mg/kg body weight), DSS disease control, and vehicle control [* represents significantly greater than all other groups (p <0.03)]. Data represent means ± SE (n = 8). B. Example of flow cytometery gating: upper left quadrant represents the classic monocyte population (CD14+/CD16-), whereas the upper right quadrant represents the inflammatory monocyte population (CD14+/CD16+).

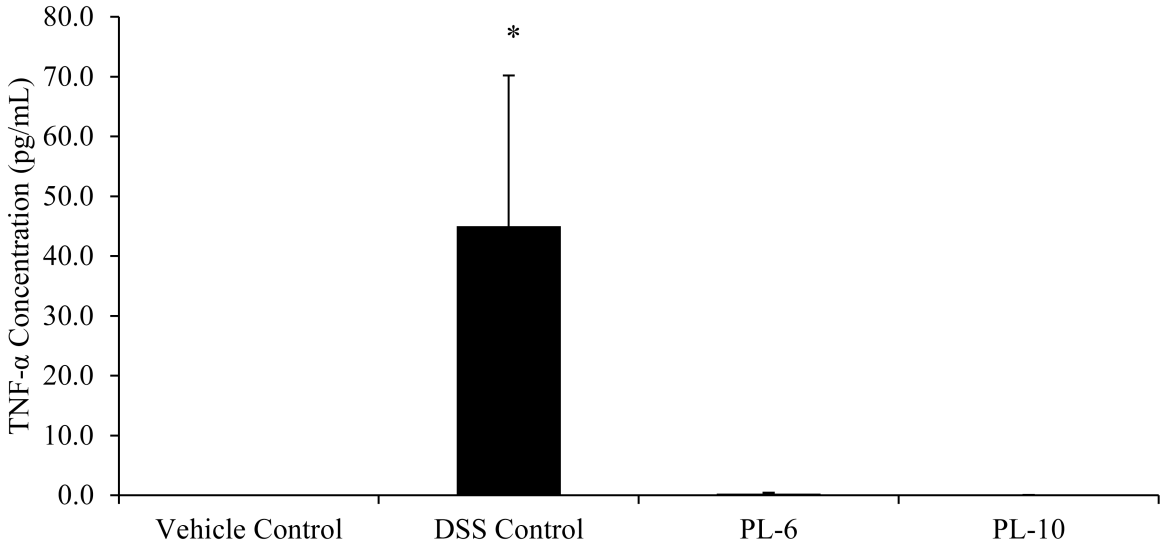

Figure 3.

Serum TNF-α concentration in plumbagin (**1**) treatment groups (PL-6 and 10 mg/kg body weight), DSS-disease control, and vehicle control (chronic model). Significant differences of treatment by **1** with respect to DSS disease control and vehicle control are indicated ($p < 0.05$). Data represent means ± SE ($n = 10$).

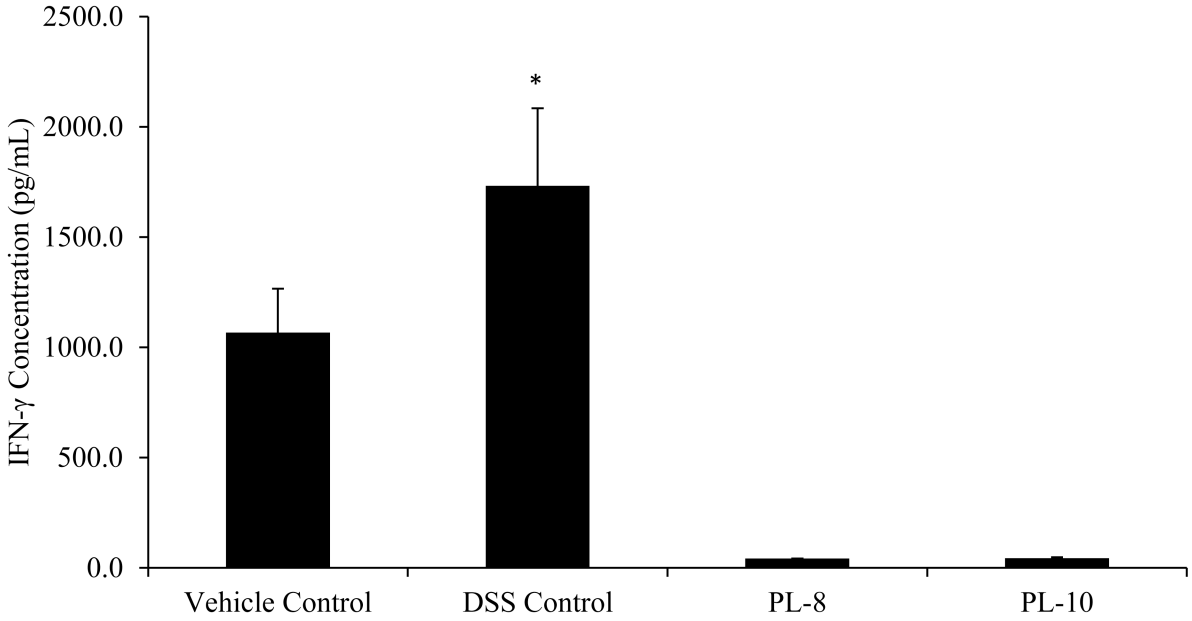

Figure 4.

Serum IFN-γ concentration in plumbagin (**1**) treatment groups (PL-8 and 10 mg/kg body weight), DSS disease control, and vehicle control (chronic model). Significant differences of treatment by **1** with respect to DSS disease control and vehicle control are indicated ($p < 0.05$). Data represent means ± SE ($n = 8$).

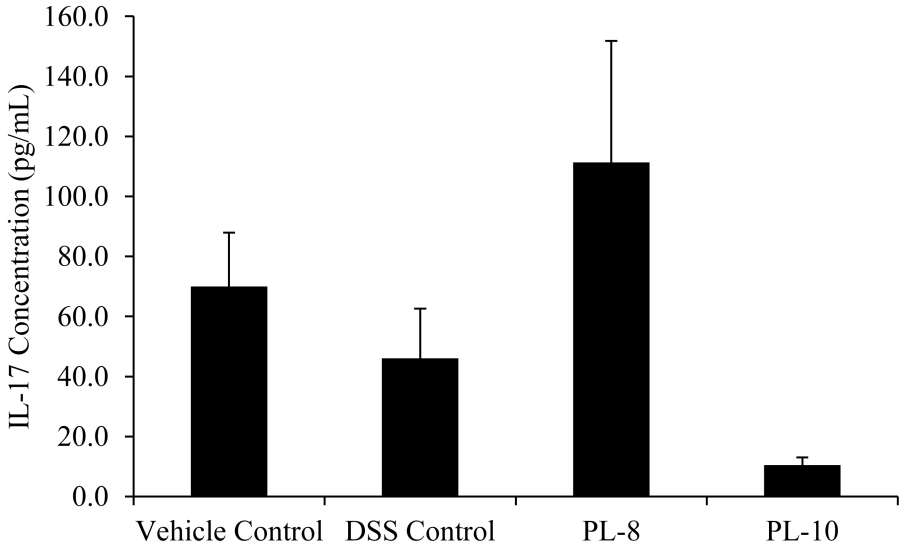

Figure 5.

Serum IL-17 concentration in plumbagin (**1**) treatment groups (PL-8 and 10 mg/kg body weight), DSS disease control, and vehicle control (chronic model). Significant differences of treatment by **1** with respect to DSS disease control and vehicle control are indicated ($p < 0.05$). Data represent means ± SE ($n = 10$).

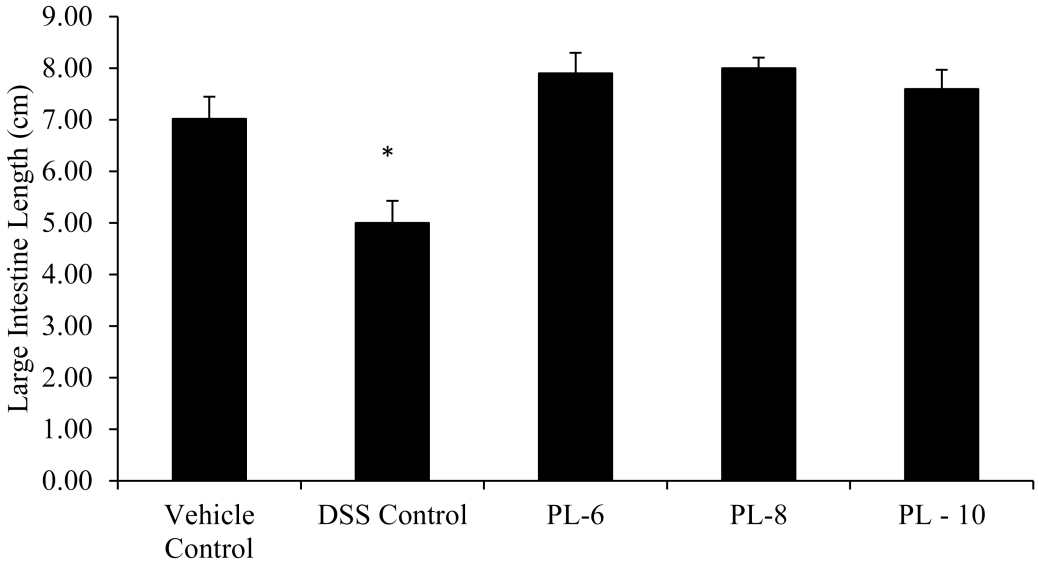

Figure 6.

Length of colon in plumbagin (**1**) treatment groups (PL-6, -8, -10 mg/kg body weight), DSS disease control, and vehicle control (chronic model). Significant differences of treatment by **1** with respect to DSS disease control and vehicle control are indicated (p <0.05). Data represent means ± SE (n = 10).

Figure 7 (A, B).

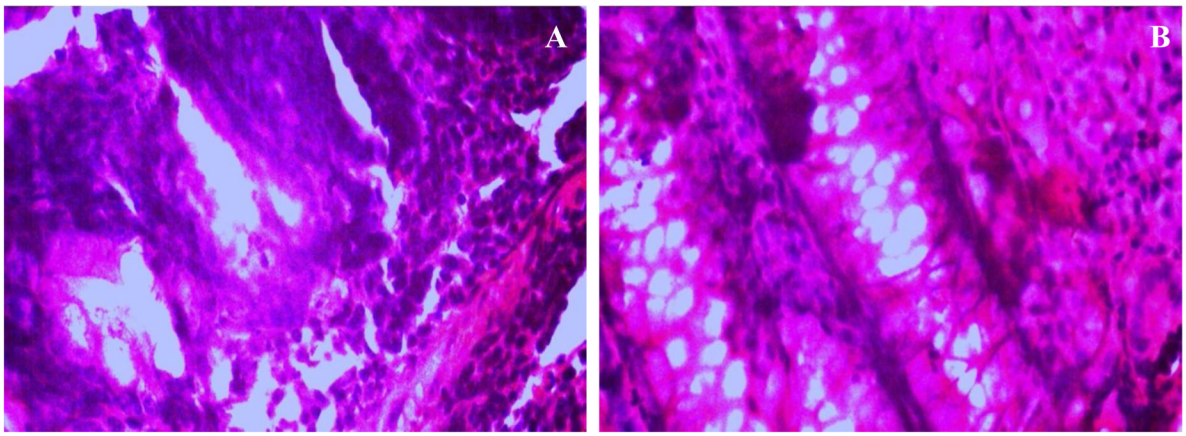

Figure 7.
Histological colorectal sections (x 400) representing: (A) DSS disease control colon with disruption of crypts, goblet cells, and aggregation of numerous monocytes; (B) the colon after treatment with **1** (10 mg/kg body weight) showing the restoration of crypts, goblet cells, and reduction in monocytes.

Table 1
Disease Activity Index (DAI) Scores Based on Disease Marker Intensities

disease activity index (DAI)	% change in body weight of mice	colorectal length (cm)	diarrhea (stool consistency)	fecal occult blood, prolapse
4	>10-15% loss	>6.5 – 7.0 cm	3	dark blue; prolapse+
3	>5-10% loss	>7.0 – 7.5 cm	2	moderately blue
2	>0-5% loss	>7.5 – 8.0 cm	1	faintly blue
1	0-5% gain	>8.0 – 8.5 cm	0	negative