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Preventive effect of carob (*Ceratonia siliqua* L.) in a dextran sulfate sodium-induced ulcerative colitis in rat

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Abstract

Inflammation and oxidative stress are a common mechanism of many gastrointestinal diseases such ulcerative colitis. Polyphenols are micronutrients with antioxidant and anti-inflammatory properties and may play an interesting role in the prevention of intestinal inflammation. In this context, we examined the protective effect of carob pods aqueous extract (CPAE) against dextran sulfate sodium (DSS)-induced sub-acute experimental ulcerative colitis in rat model. Colon inflammation was induced in rat by oral administration of synthetic DSS (5%) in the drinking water during 7 days. However, CPAE (50 and 100mg/Kg, *b.w.*) was given by oral administration for 21 days. At the end of the experimental period, colon and plasma were taken for lipid peroxidation and cytokines determination as well as myeloperoxidase (MPO) and antioxidant enzyme activities. Our result indicated that DSS caused severe histopathological damages in the colon mucosa. The lesions were associated with increased MPO activity, cytokines and oxidative damage. The CPAE treatment restored dose-dependent body weight gain, prevented colonic shortening and reduced the colonic severity lesions and biochemical alterations. It was concluded that CPAE consumption limits DSS-induced colonic damage in rats. The antioxidant and anti-inflammatory properties of polyphenols may play a role in this effect.

Key words: CPAE; dextran sulfate sodium; ulcerative colitis; inflammation; oxidative stress.

Running title: Effect of AECP on DSS-induced ulcerative colitis.

1. Introduction

The chronic inflammatory disorders of the intestinal mucosa, with organ-susceptibility for the colon, is often characterized by bloody diarrhea, tenesmus and abdominal pain.[1,2] The DSS model produces inflammation limited to the colonic mucosa that is more closely related to human ulcerative colitis which shares numerous clinical, biochemical and histological feature.[3,4] However, a number of medicinal plants have been investigated for their possible anti-inflammatory and anti-oxidative activities.

Ceratonia siliqua L. is an evergreen tree cultivated or naturally grown in the Mediterranean area. In Tunisia, it occurs in a spontaneous state, although it was cultivated for a long time for human and animal food.[5] The mature fresh fruit of carob is made up of about 90% of pod (known as kibble) and 10% of seeds.[6] These later, covered with a tight-fitting brown coat, contain a white and translucent endosperm (containing galagtomannans), also called carob gum. Sugars contained in the pods are mainly composed of sucrose, fructose and glucose. The pods also contain a high amount of dietary fiber and phenolic compounds.[7] these molecules are a very heterogeneous group and include simple phenolic acids, cinnamic acid and its derivatives, flavonoids, isoflavones, lignans, anthocyans and tannins.[8] Several epidemiological studies show the protective effects of dietary fiber intake on intestinal inflammation, colorectal cancer and cardiovascular disease.[9,10] Also, phenolic compounds are an important bioactive component of medicinal plant extract exhibiting various pharmacological properties[11] such antioxidative and anti-inflammatory properties and may play an interesting role in the prevention against intestinal inflammation. Flavonoids are known to act on the inflammatory response via many routes and block molecules like cyclooxygenase (COX), nitric-oxide synthase (iNOS), cytokines, nuclear factor-κB and matrix metalloproteinases. In addition, flavonoids have strong antioxidant, free radical scavengers that donate hydrogen,

inhibit lipid peroxidation [12] and also metal ion chelators. These groups of phytochemicals are known to play some beneficial roles in the prevention of many oxidative and inflammatory diseases.[13,14] Gallotannins exhibit biological activities including antimicrobial, antiviral, anti-inflammatory to anticancer and antiviral properties.[15] The mechanisms underlying the anti-inflammatory effect of tannins include the scavenging of radicals, as well as inhibition of the expression of inflammatory mediators, such as some cytokines, inducible nitric-oxide synthase, and COX-2.[16,15] Tannins can also bind to some free radical producing enzymes forming an insoluble tannin-protein complex (astringent characteristic), with catalytic metallic ions making it unavailable to initiate oxidation reaction, and inhibiting lipid peroxidation process.[17]

In this respect, the present study was designed to elucidate the protective effect of carob pods treatment on DSS-induced damage in the rat colon mucosa.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (weighing 170-200 g; housed five per cage) were obtained from "Le Genest-St-Isle, France" and used in accordance with the local ethic committee of French University for use and care of animals in conformity with the NIH recommendations. Before any experience, all animals were kept one week under the same laboratory conditions of temperature (22±2 °C), relative humidity (70±4%) and a 12 h light/dark cycle and received a nutritionally standard diet and tap water.

2.2. Extract preparation

The mature carob pods were collected from the region of Tabarka, (North-western of Tunisia) during October 2013. The Voucher specimens have been deposited with the herbarium of the Higher Institute of Biotechnology of Beja and also in Department of Biological Sciences, Faculty of Science of Tunis. The plant material was dried in an incubator at 50 °C during 72 h and powdered in an electric blender (MoulinexOvatio 2, FR). Powder mixture containing carob pulp (90 %) and seeds (10 %) was dissolved in double distilled water and filtered through a colander (0.5 mm mesh size). Finally, the carob pods aqueous extract was immediately used for *in vivo* experiments.

2.3. Experimental Design

Rats were randomly divided into four groups of 10 each: Control, DSS and DSS + two doses of CPAE (50 and 100 mg/kg, *b.w.*). Animals were per orally pre-treated with CPAE during three weeks and intoxicated by DSS in the drinking water for 7 days (day 14 to 21). Body weight and food intake were monitored daily for 21 days. 24 hours, after the last carob administration and an overnight fast, animals were anaesthetized by intraperitoneal

administration of sodium pentobarbital (40 mg/kg body weight) and sacrificed by decapitation.

2.4. Colon and plasma sampling

After sacrifice, the colons were quickly removed and washed in 0.9% NaCl and the lengths were measured. The mucosal colon specimens were then placed in a phosphate buffered saline (PBS) solution, homogenized and centrifuged for 15 min at 9000 g. Supernatants were stored at –80°C for the determination of biochemical parameters. On the other hand, blood was likewise collected in heparinized tubes. After centrifugation at 3 000 × *g* during 15 min, plasma was also stored at –80°C.

2.5. Histopathological examinations

The section for histological examination was fixed in alcohol-formalin-acetic acid solution and embedded in paraffin. Three 5 µm sections were cut serially. The next three sections were cut at a distance of 100 µm. A third set of sections was then cut after 100 µm. All sections were stained with haematoxylin-eosin-safran and then evaluated.[18] Stained cells were examined with numerical aperture objective under a Zeiss LSM510 confocal microscope (Carl Zeiss, Heidelberg, Germany), and the images were imported into an LSM image browser (Carl Zeiss) for analysis.

2.6. Measurement of lipids peroxidation and MPO activity

The rate of lipoperoxidation in the colonic mucosa samples was estimated by determination of malondialdehyde (MDA) using the Thiobarbituric Acid Reactive Substances (TBARS) test.[19] MPO activity was assessed by using the H_2O_2-dependent tetramethylbenzedine (TMB) oxidation assay at 650 nm.[20]

2.7. Determination of colonic and plasmatic antioxidant enzyme activities and cytokines

Superoxide dismutase (SOD) activity was estimated by the inhibition of nicotinamide adenine dinucleotide (reduced)-phenazinemethosulphate-nitrobluetetrazolium reaction systemas adapted by Kakkar et al.,[21] and the results have been expressed as units (U) of SOD activity/mg protein. Catalase (CAT) was estimated by method of Aebi [22] and the results are expressed as nmol/min/mg protein. GPx activity was measured by the procedure of Flohé and Günzler.[23]

TNF-α and IL-1β levels were quantified in the supernatants of colon by standard cytokine-specific rat ELISA kits and expressed in pg per mg of proteins. The assays were performed in duplicate.

2.8. Statistical Analysis

The data were analyzed by one-way analysis of variance (ANOVA) and were expressed as means ± standard error of the mean (S.E.M.). The data are representative of 10 independent experiments. All statistical tests were two-tailed, and a p value of 0.05 or less was considered significant.

3. Results

3.1. Food intake, body weight gain and colon length

Table 1 shows the effect of repeated administration of CPAE on body weight gain, food intake and colon length of inflamed rats. At the end of treatment, the DSS treated rats showed a significant decrease of body weight gain (13%) compared to the controls. The administration of CPAE during 3 weeks at the doses of 50 and 100 mg/kg of body weight induced a significant dose-dependent weight gain (8% and 14%) as compared to DSS group. However the food intake was also decreased significantly in inflamed rats (10%) when compared to control. The pretreatment with plant extract at 50 and 100 mg/kg of body weight significantly increased food intake (5% and 12%) compared to DSS group. The colitis induced also a reduction in colon length (8%). While, CPAE consumption prevented the colon shortening (Table 1).

3.2. Histopathological examination

The colon of rats receiving DSS (5%) (Fig. 1b) presented epithelium destruction, polymorphonuclear infiltration and oedema throughout the mucosa and sub-mucosa compared to normal tissue sections (Fig. 1a). Pre-treatment with carob pods also protected against lesions promoted by DSS (5%) and mucosa against epithelial and glandular destruction. The pre-treatment with CPAE (100mg/kg, *b.w.*) for 21 days showed histological aspects of the colonic mucosa similar to the group without lesions (Fig. 1D). However, the histological score was lower in carob-DSS rats than DSS animals (Fig. 1E).

3.3. Colonic and plasmatic MDA content and MPO activity

Induction of colitis by DSS causes a significantly increase of MDA content in the colon and plasma when compared to the control group. However, carob pods aqueous extract consumption significantly and dose-dependently decreased colonic and plasmatic MDA content in inflamed rats (Fig. 2).

MPO activity, as a marker of inflammation, was significantly increased in the colitis group (DSS) compared to control. Carob pre-treatment significantly protected against DSS-induced colonic inflammation (Fig. 3).

3.4. Effect of CPAE on DSS-induced antioxidant enzyme activities alteration

The antioxidant enzyme activities recorded show that the SOD and catalase activity are significantly increased in the colitis group (DSS) compared to control. However, GPx activity was not significantly different between groups. The pre-treatment of rats with carob pods aqueous extract significantly decreased DSS-induced increase of antioxidant enzyme activities to near control levels with the highest dose (Fig. 4, 5 and 6).

3.5. Colonic cytokines levels

Table 2 summarizes colonic TNF-α and IL-1β concentrations. DSS (5%) administration significantly increased cytokines levels. This increase of TNF-α and IL1β content in colon was significantly abrogated by carob extract pretreatment in a dose-dependent manner.

4. Discussion

In the present study, we examined the effect of carob pods (*Ceratonia siliqua* L.) aqueous extracts against DSS-induced oxidative stress, myeloperoxidase activity and cytokines levels increase. Our phytochemical study firstly revealed that CPAE is rich in total polyphenols, flavonoids and condensed tannins.[24], we also found that CPAE presents, using the DPPH and ABTS radical-scavenging assays, a high scavenging capacity, albeit lesser than ascorbic acid which was used as a reference molecule.[25]

The HPLC technique, allowed to the identification of many phenolic compounds with pyrogallol, catechin, tannic acid, gallic acid and ferulic acid as main compounds in the pulp and tannic acid, pyrogallol, catechin and vannilic acid in the seeds.[26] However, previous studies have reported that these molecules are well known for their antioxidant properties. [27,28] Our data are in agreement with those of Corsi et al., who have mainly identified gallic acid, and also (-)-epigallocatechin-3-gallate (EGCG) and (-)-epicatechin-3-gallate (ECG) in an infusion of carob pods with boiling water, and several authors referred also to the presence of hydrolysable tannins (gallotannins and ellagitannins) in carob pods. These results are also in agreement with those of Owen et al.,[8] who have identified polyphenols as tannins, flavonoids and phenolic acids (such as gallic acid, cinnamic acid and p-coumaric acid), flavone glycosides (such as quercetin-3-O-α-L-rhamnopyranoside) and hydroxytyrosol.[29,30] Such compounds have been shown to possess an anti-inflammatory and oxidative stress effects.[31-36]

The bioaccessibility of polyphenolic compounds has been the subject of many recent reviews. Some reviews focused on polyphenols from individual plant sources, such as tea [37] and cocoa.[38] In fact, the bioavailabilites of catechin and tannic acid were evaluated *in vitro* in ligated rat small intestine segments. Although both compounds were absorbed by the intestinal wall (uptake: tannic acid 50%, catechin 30%), only catechin was shown to traverse

the gut in low amounts. [39] Phenolic acids with small-molecular weight such as gallic acid and isoflavones are easily absorbed through the tract, as well as flavones, catechins, and quercetin glucosides. [40]

Colitis induced by daily oral administration of DSS (5%) is one of the most widely used models for inflammatory bowel disease (IBD). DSS causes injury mainly in the distal colon [4], and lesions are accompanied by mucosal infiltration of neutrophils.[41]Although the exact mechanism of action of DSS is not fully understood. However, it has been shown that DSS intoxication increases colonic mucosal permeability, leading to destruction of the mucosal barrier function and inflammatory reactions.[42] The DSS model of colitis has been shown to cause also significant oxidative stress in rat colon [43,44]. However, the antioxidant compounds such as green tea polyphenols,[44] Antioxidant Biofactor® [45] and dehydroepiandrosterone [46] have been shown to reduce the severity of DSS colitis.

The conditions of DSS administration (5% for 7 days) were chosen to induce a mild-to-moderate colitis that could be more amenable to nutritional prevention.

In these conditions, DSS significantly reduced food intake, body weight gain and colon length. The carob pods aqueous extract consumption in DSS-treated rats kept these alterations and lead to control values at 100mg/kg. The protective effects of pure polyphenols or polyphenol-rich extracts on colon length have been previously observed in colitis rats.[47,48]

Histological examination of colon samples from DSS-treated rats showed epithelium destruction with glandular dilatation, edema and polymorphonuclear infiltration, as previously reported.[49-51] These alterations were significantly and dose-dependently corrected by carob pods aqueous extract pretreatment. Also, beneficial effects of various polyphenols on colonic histological structure have often been reported. Purified verbascoside improved the histopathological lesions induced by intrarectal administration of 2,4-dinitrobenzene sulfonic

acid (DNBS),[52] while diet supplementation with resveratrol or curcumin also decreased mucosal erosion in trinitrobenzene sulfonic acid (TNBS) and DSS models.[50,53,54]

Moreover, increased MDA level by DSS (5%) induced lipid peroxidation, which is responsible for some of the overall effect, leading to release of cell contents, cross-linking of protein, nucleic acid molecules and cell toxicity and death.[55] In our work, colonic and plasmatic MDA content decreased significantly after treatment of rats by the CPAE. Studies carried out with herbal extracts such as a *Punica granatum* [56], lemon verbena infusion,[57] resveratrol [58] or purified polyphenols such as luteolin [59] have also reported a decrease of colonic lipoperoxidation. MPO is used as a marker of inflammatory response in the mucosa, and its activity in the colon is linearly related to neutrophils infiltration.[60] DSS-induced inflammation resulted in an increase of colonic and plasmatic MPO activity that was significantly and dose-dependently prevented by carob pods. MPO is used as a marker of inflammatory response in the mucosa, and its activity in the colon is linearly related to neutrophils infiltration. [60] DSS-induced inflammation resulted in an increase of colonic and plasmatic MPO activity that was significantly and dose-dependently prevented by CPAE. In this context, we showed that *in vitro* CPAE inhibits the MPO activity in a concentration-dependent manner. This inhibition by carob extracts has therefore, the ability to reduce the production of hypochlorous acid (HOCl) from H_2O_2 and could attenuate the inflammatory reactions.[26]

During inflammatory processes, neutrophil degranulation releases a large amount of MPO which metabolizes H_2O_2 into HOCl.[61] Moreover, phagocytes (neutrophils and macrophages) infiltrated into the mucosa are activated by pro-inflammatory factors that will stimulate ROS production and particularly $O_2^{\bullet-}$ overproduction, thus inducing oxidative stress.[62] Oxidants play a direct role in the chronic inflammatory process by increasing the number of neutrophils and macrophages that induce a self-sustaining phlogogenic loop.[63] In addition, it has been

shown that the activated state of NAPDH oxidase can produce more ROS and superoxide, which leads to further colonic tissue injury. Therefore, maintaining the balance of the antioxidant/oxidant system is critical for preventing increased oxidative stress and inflammation and, consequently, ulcerative colitis. In our study, we showed that the CPAE (pulp and seeds) inhibits in a concentration dependent manner ROS, superoxide anion and H_2O_2 production. We also, examined the effect of CPAE and its ability to inhibit the phosphorylation of p47phox Ser-328 which induces the inhibition of NADPH oxidase activity.[26]

In our research, SOD and CAT activities were significantly increased by DSS intoxication. However, preventive carob pods consumption decreased antioxidant enzymes activity. Because polyphenols are known for their antioxidant properties,[64] we evaluated the antioxidative defenses that could play an important role in macromolecule protection against oxidation.

A helping effect of polyphenols on SOD and catalase activity has previously been reported in various models. Besides, a stimulating effect of polyphenols on antioxidant enzymes activity has previously been reported in various models. In the azoxymethane carcinogenesis model, supplementation with the flavonoids luteolin induced an increase of SOD activity. However, aqueous extract of carob pods did not significantly change other antioxidative enzymes such as GPx activity in DSS-treated rats. Little is known about the effects of polyphenols on GPx activity during colitis, except that GPx activity is increased by luteolin in azoxymethane-induced colon carcinogenesis.[59]

Colitis is usually associated with the production of pro-inflammatory cytokines by immune cells.[65] IL-1β and TNF-α mediate the neutrophils migration observed in several experimental models and also in human inflammatory disease.[36]

In our study, DSS significantly increased the IL-1β and TNF-α levels in colon that were corrected by aqueous extract of carob pods. These beneficial effects are mainly attributed to polyphenols. For this instance, verbascoside decreased pro-inflammatory cytokine levels (TNF-α, IL-1β) in various models of murine colitis.[52,49] Moreover, all parameters indicating the presence of inflammation and oxidative injury were inhibited by the carob pods aqueous extract, suggesting that carob may have a potent anti-inflammatory effect on the inflamed colonic tissue and plasma.

5. Conclusion

In conclusion, our results demonstrate that carob pods aqueous extract presents a protective effect against DSS-induced experimental colitis. The reversal of mucosal injury seems to be mainly related to the antioxidant and anti-inflammatory properties of our plant extract.

Competing interests

The authors declare that they have no competing interests.

Abbreviations: CPAE, carob pods aqueuos extract; CPAE-50, CPAE 50mg/kg; CPAE-100, CPAE 100mg/kg; CAT, catalase; DSS, dextran sulfate sodium; GPx, glutathione peroxidase; H_2O_2, hydrogen peroxide; IL-1β, interleukin-1 beta; MDA, malondialdehyde; MPO, myeloperoxidase; Nacl, sodium chloride; SOD, superoxide dismutase; TNFα, tumor necrosis factor alpha.

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Legends of figures

Figure 1: Histological analysis of colonic mucosa: resection of control (A) colons showed normal mucosa with intact epithelial surface whereas DSS-induced colitis rats (B) presented massive epithelial and glandular destruction, polymorphonuclear infiltration, ulceration and submucosal inflammation. Carob pods aqueous extract consumption (C, D) protected mucosa against epithelial and glandular destruction. (Original magnification x 10). Histological score (E): Values are means ± SEM (n = 10).

Figure 2: Sub-acute effect of carob pods aqueous extract (CPAE) on DSS-induced changes in plasma and colonic mucosa MDA level in rats. Animals were pre-treated with various doses of CPAE (50 and 100mg/kg, *b.w.*, *p.o.*), or bi-distilled water, challenged with oral administration of DSS (5%) or NaCl 9‰ for 7 days. *: $p < 0.05$ compared to control group and $^{\#}$: $p < 0.05$ compared to DSS group. Values are means ± SEM (n = 10).

Figure 3: Sub-acute effect of carob pods aqueous extract (CPAE) on DSS-induced changes in plasma and colonic mucosa MPO activity in rats. Animals were pre-treated with various doses of CPAE (50 and 100mg/kg, *b.w.*, *p.o.*), or bi-distilled water, challenged with oral administration of DSS (5%) or NaCl 9‰ for 7 days. *: $p < 0.05$ compared to control group and $^{\#}$: $p < 0.05$ compared to DSS group. Values are means ± SEM (n = 10).

Figure 4: Sub-acute effect of carob pods aqueous extract (CPAE) on DSS-induced changes in plasma and colonic mucosa SOD activity in rats. Animals were pre-treated with various doses of CPAE (50 and 100mg/kg, *b.w.*, *p.o.*), or bi-distilled water, challenged with oral

administration of DSS (5%) or NaCl 9‰ for 7 days. *: $p < 0.05$ compared to control group and #: $p < 0.05$ compared to DSS group. Values are means ± SEM ($n = 10$).

Figure 5: Sub-acute effect of carob pods aqueous extract (CPAE) on DSS-induced changes in plasma and colonic mucosa CAT activity in rats. Animals were pre-treated with various doses of CPAE (50 and 100mg/kg, *b.w.*, *p.o.*), or bi-distilled water, challenged with oral administration of DSS (5%) or NaCl 9‰ for 7 days. *: $p < 0.05$ compared to control group and #: $p < 0.05$ compared to DSS group. Values are means ± SEM ($n = 10$).

Figure 6: Sub-acute effect of carob pods aqueous extract (CPAE) on DSS-induced changes in plasma and colonic mucosa GPx activity in rats. Animals were pre-treated with various doses of CPAE (50 and 100mg/kg, *b.w.*, *p.o.*), or bi-distilled water, challenged with oral administration of DSS (5%) or NaCl 9‰ for 7 days. *: $p < 0.05$ compared to control group and #: $p < 0.05$ compared to DSS group. Values are means ± SEM ($n = 10$).

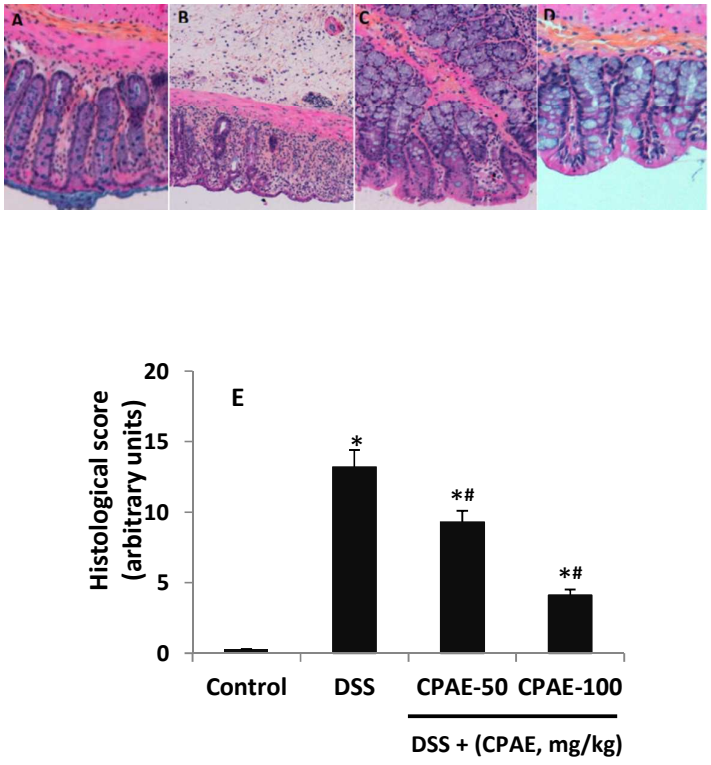

Figure 1

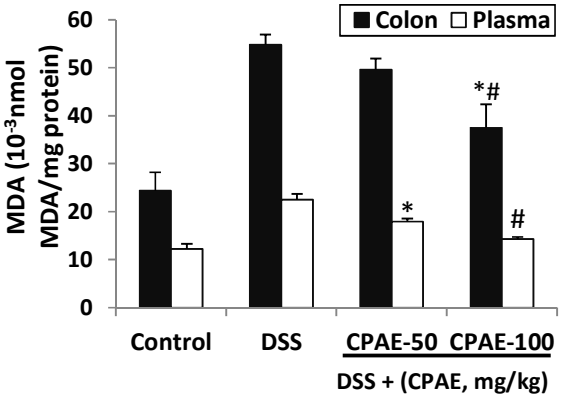

Figure 2

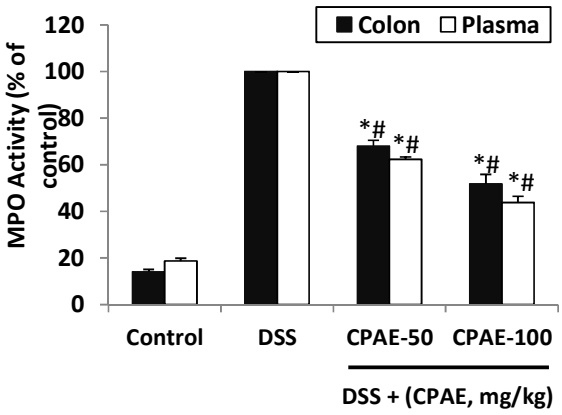

Figure 3

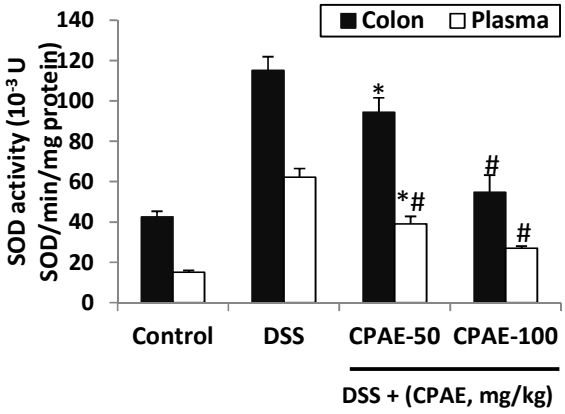

Figure 4

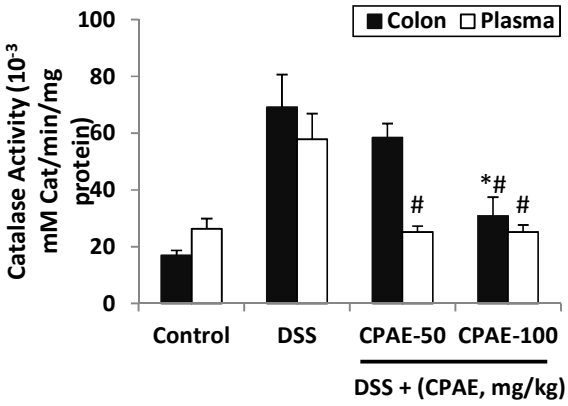

Figure 5

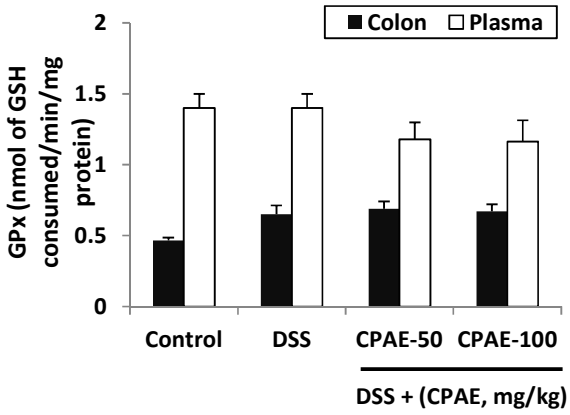

Figure 6

Table 1

Food intake, body weight gain and colon length.

Parameters	Food intake (g)	Body weight gain (%)	Colon length (cm)
Control	200.1 ± 6.64	29.71 ± 1.81	18.6 ± 2.23
DSS	180.1 ± 4.27*	16.32 ± 0.52*	12.8 ± 0.56*
DSS + CPAE-50	190.4 ± 1.47*$^{\#}$	24.00 ± 1.44*$^{\#}$	15.00 ± 1.41*$^{\#}$
DSS + CPAE-100	205 ± 2.74$^{\#}$	30.52 ± 2.21$^{\#}$	16.02 ± 0.33$^{\#}$

Values are means ± SEM (n = 10). Control group; DSS, DSS (5%) group; DSS + CPAE-50, DSS (5%) + Carob pods aqueous extract (50mg/Kg, *b. w.*) group; DSS + CPAE-50, DSS (5%) + Carob pods aqueous extract (100mg/Kg, *b. w.*) group. *: $p < 0.05$ compared to control group and $^{\#}$: $p < 0.05$ compared to DSS group.

Table 2

Colonic cytokines levels (pg/mg proteins).

Groups	TNF-α	IL-1β
Control	2.70 ± 0.23	55.40 ± 3.46
DSS	12.22 ± 1.00 [b]	166.03 ± 5.63 [b]
DSS + CPAE-50	8.30 ± 0.51 [a,b]	126.00 ± 3.44 [a,b]
DSS + CPAE-100	6.14 ± 0.74 [a,b]	78.00 ± 0.23 [a,b]

The values are means ± SEM (n = 10).Control group; DSS, DSS (5%) group; DSS + CPAE-50, DSS (5%) + Carob pods aqueous extract (50mg/kg, *b. w.*) group, DSS (5%) + CPAE-100, DSS (5%) + Carob pods aqueous extract (100mg/kg, *b. w.*) group. Data was analyzed by Statview ANOVA. **[a]**: $p < 0.01$ when compared to DSS group, **[b]**: $p<0.01$ when compared to control group.

Carob Pods Aqueous Extract (CPAE)

DSS

AECP pre-treatment protected against DSS-induced inflammation and oxidative stress

Food intake, Body weight gain and Colon length

Histological Analyzes (Colon)

Biochemical Analyzes (oxidant, antioxidant status and cytokins)