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Dietary protocatechuic acid ameliorates dextran sulphate sodium-induced ulcerative colitis and hepatotoxicity in rats

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Abstract

The present study investigated the antioxidant and anti-inflammatory effects of dietary protocatechuic acid (PCA), a simple hydrophilic phenolic compound commonly found in many edible vegetables, on dextran sulphate sodium (DSS)-induced ulcerative colitis and its associated hepatotoxicity in rats. PCA was administered orally at 10 mg/kg to dextran sulphate sodium exposed rats for five days. The result revealed that administration of PCA significantly (p < 0.05) prevented the incidence of diarrhea and bleeding, the decrease in the body weight gain, shortening of colon length and the increase in colon mass index in DSS-treated rats. Furthermore, PCA prevented the increase in the plasma levels of pro-inflammatory cytokines, markers of liver toxicity and markedly suppressed the DSS-mediated elevation in colonic nitric oxide concentration and myeloperoxidase activity in the treated rats. Administration of PCA significantly protected against colonic and hepatic oxidative damage by increasing the antioxidant status and concomitantly decreased hydrogen peroxide and lipid peroxidation levels in the DSS-treated rats. Moreover, histological examinations confirmed PCA chemoprotection against colon and liver damage. Immunohistochemical analysis showed that PCA significantly inhibited cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) protein expression in the colon of DSS-treated rats. In conclusion, the effective chemoprotective role of PCA in colitis and the associated hepatotoxicity is related to its intrinsic anti-inflammatory and anti-oxidative properties.

Keywords: Protocatechuic acid, Anti-oxidative, Anti-inflammatory, Colitis, Rats
INTRODUCTION

Ulcerative colitis is a human inflammatory disease of the colonic mucosa known to be associated with hyper-inflammation of the gastrointestinal tract and a disturbance of mucosal immune respons.\(^1\) Ulcerative colitis patients reportedly generate high levels of reactive oxygen species (ROS) and proinflammatory cytokines in their colonic mucosa.\(^2,3\) The existence of a close relationship between ulcerative colitis and various hepatic disorders has been reported.\(^4,5\) About 5-10% of patients suffering from inflammatory bowel diseases have been reported to develop hepatobiliary disorders.\(^6\) The proximity of gastrointestinal tract to the hepatobiliary system predisposes the liver and biliary system the direct targets for damage in ulcerative colitis condition.\(^5\) Therapeutic agents that would simultaneously control both inflammatory immune response and oxidative stress are needed in the treatment ulcerative colitis.\(^3\)

Protocatechuic acid (PCA, 3,4-dihydroxybenzoic acid, figure 1A) is a simple hydrophilic phenolic compound commonly found in many edible vegetables, fruits and nuts.\(^7\) The beneficial human health effects of PCA have been demonstrated experimentally to involve multiple biochemical mechanisms. The anti-oxidative effect of PCA has been shown to be 10-fold higher than that of \(\alpha\)-tocopherol.\(^8\) It has also been reported to be a potent chemoprotective agent against trans fatty acid-induced hepatic steatosis in mice.\(^9\) It exhibited antioxidant and anti-hyperlipidaemic activities in streptozotocin diabetic rats.\(^10\) The anti-inflammatory and analgesic activities of PCA in both rats and mice has been reported.\(^11\) The anti-ageing property of PCA was demonstrated by its ability to normalize age-associated alterations in spleen and liver of senescent mice.\(^12\) The anti-apoptotic and pro-survival effect of PCA on hypertensive hearts of rats have been reported.\(^13\) Moreover, PCA decreased both activity and expression of
cyclooxygenase (COX)-2 as well as reduced the release of interleukin (IL)-1beta, IL-6, tumor necrosis factor-alpha and prostaglandin E(2) in brain of mice treated with D-galactose.\textsuperscript{14} In general, the activation of these inflammatory mediators which are known cause tissue damage and are thought to be critical events in the pathogenesis of ulcerative colitis.\textsuperscript{3} However, there is a dearth of information regarding the effect of PCA on inflammatory bowel diseases such as ulcerative colitis.

Considering the previous documentation on PCA to suppress oxidative stress and inflammation in different experimental models of organ toxicity, the present study was designed to investigate its effect on dextran sulfate sodium (DSS)-induced colitis which is a well established experimental model with pathologic features of human colitis.\textsuperscript{15,16} In addition, the influence of PCA on colitis mediated hepatotoxicity, which till date is unreported in the literature, was explored in the experimental rats.
MATERIALS AND METHODS

Drugs and chemicals

Dextran sulphate sodium salt (DSS) was obtained from TdB Consultancy (Uppsala, Sweden). Protocatechuic acid (PCA), trichloroacetic acid (TCA), thiobarbituric acid (TBA), 1-chloro-2,4-dinitrobenzene (CDNB), 5',5'-dithiobis(2-nitrobenzoic acid) (DTNB), hydrogen peroxide (H₂O₂), reduced glutathione and xylenol orange were procured from Sigma Chemical Company (St. Louis, MO, USA). Goat polyclonal antibody against cyclooxygenase-2 (COX-2) and rabbit polyclonal antibody against inducible nitric oxide synthase (iNOS) were procured from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies in VECTASTATIN kit were purchased from Vector Labs (Burlingame, CA, USA). All other reagents were of analytical grade and were obtained from the British Drug Houses (Poole, Dorset, UK).

Animal model

Forty adult male Wistar rats (10 weeks old; 182 ± 5 g) obtained from the Department of Biochemistry, University of Ibadan, Ibadan, Nigeria, were used for the present investigation. They were housed in plastic cages placed in a well-ventilated rat house, provided rat pellets and water ad libitum and subjected to natural photoperiod of 12-hr light : 12-hr dark. All the animals received humane care according to the criteria outlined in the ‘Guide for the Care and Use of Laboratory Animals’ prepared by the National Academy of Science and published by the National Institute of Health. The experiment was performed according to the guidelines and approval of University of Ibadan Ethical Committee.
Experimental protocol

The rats were randomly assigned to four groups of ten rats per group. Group I rats received corn oil at 2 mL/kg orally once daily for 10 consecutive days and served as control. Group II rats were treated orally with PCA alone at 10 mg/kg\textsuperscript{12} in corn oil once daily for 10 consecutive days. Group III rats were maintained on normal rat chow for the first five days before exposure to DSS (molecular weight 37–40 kD; 5% w/v)\textsuperscript{17} in drinking water throughout the last five days of the study. Group IV rats were pre-treated with PCA at 10 mg/kg in corn oil for the first five days followed by a daily administration of both DSS (5% w/v) and PCA for the last five days of the experiment. We chose 10 mg/kg PCA because it was the highest effective dose among the various doses (5, 10, and 20 mg/kg) investigated in our preliminary studies (data not shown).

Twenty-four hours after the last treatment, five milliliters of blood was drawn from the retro-orbital venous plexus of the animals into vials containing heparin as an anticoagulant. Plasma samples were separated from blood cells by centrifugation at 3000 x g for 10 minutes. The severity of disease was calculated by scoring 3 parameters (weight loss, stool consistency, and gross bleeding) according to the method previously described.\textsuperscript{18} Rectal bleeding was scored 0 for normal; 2 for occult bleeding; 4 for dross bleeding while stool consistency was scored 0 for normal; 2 for loose; 4 for diarrhea. The colon mass index (CMI), calculated as the ratio of colon weight in milligrams to total body weight in grams, was used to evaluate the degree of colonic edema and severity of inflammation. All the animals were sacrificed by cervical dislocation and the colons and livers were immediately excised, rinsed with ice-cold phosphate-buffered saline and processed for biochemical and histological analysis.
Plasma biochemistry

Plasma activities of aspartate and alanine aminotransferases (AST and ALT) and alkaline phosphatase (ALP) were estimated using commercially available kits (Randox Laboratory Limited, UK). The plasma concentrations of IL-1β, IL-6 and TNF-α were quantified with the aid of DMM 9602 Microplate Reader (China) using an ELISA kit (Wkea Med Supplies, China).

Determination of colonic mucosa nitrite level and MPO activity

Nitric oxide (NO) synthase activity was determined by measuring the colonic nitrites content, the stable end products of nitric oxide (NO). Colonic nitrites content were obtained using a sodium nitrite curve as standard and expressed as µM of nitrites mg⁻¹ protein.¹⁹ Myeloperoxidase (MPO) activity was determined according to the method of Granell et al.²⁰ MPO activity was expressed as µM H₂O₂ min⁻¹ mg⁻¹ protein.

Estimation of colonic and hepatic antioxidant status

The colon and liver samples were homogenized in 50 mM Tris–HCl buffer (pH 7.40) containing 1.15% potassium chloride, and the homogenate was centrifuged at 10,000 g for 15 minutes at 4 °C. The supernatant was subsequently collected for the determination of antioxidant status in the tissues. Protein concentration was determined according to the method of Lowry et al.²¹ Superoxide dismutase (SOD) activity was determined according to the method of Misra and Fridovich.²² Catalase (CAT) activity was assayed using hydrogen peroxide as a substrate according to the method of Clairborne.²³ Glutathione-S-transferase (GST) was assayed according to the method of Habig et al.²⁴ Glutathione peroxidase (GPx) activity was determined according
to the method of Rotruck et al.\textsuperscript{25} Reduced GSH was determined at 412 nm according to the method of Jollow et al.\textsuperscript{26} Hydrogen peroxide generation was measured according to the method of Wolff.\textsuperscript{27} Lipid peroxidation was quantified as malondialdehyde (MDA) according to the method described by Farombi et al.\textsuperscript{28} and expressed as µm MDA/mg protein.

**Histological evaluation**

Histological evaluation of the colon and liver of control and treated rats were carried out with the light microscope. Colon and liver biopsies were fixed in 4\% formalin, dehydrated in serial alcohol, cleared in chloroform and embedded in paraffin. Sections of 4–5 µm were cut by a microtome and stained with hematoxylin and eosin. The slides were subsequently observed under a light microscope and photographed using a digital camera. All slides were coded before examination by investigators who were blinded to control and treatment groups.

**Immunohistochemical staining of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) protein expression**

To determine the expression of COX-2 and iNOS in the colon, neutral buffered formalin fixed colons were embedded in paraffin and sectioned at a thickness of 5 µm. The sections were subsequently deparaffinized in xylene and rehydrated with graded alcohol. Antigen retrieval was carried out by immersing the slides in 10 mM citrate buffer at 95-100 °C for 25 min with subsequent peroxidase quenching in 3\% H\textsubscript{2}O\textsubscript{2}/methanol solution. The colon sections were blocked in goat serum followed by an overnight incubation at 4°C in the goat anti-COX-2 and rabbit anti-iNOS primary antibodies. Detection of bound antibody was done using appropriate HRP-conjugated secondary antibodies VECTASTAIN kit (Vector labs, USA) according to
manufacturer’s protocol. Reaction product was enhanced with 3,3’-diaminobenzidine (DAB) for 6-10 min. The sections were subsequently dehydrated in ethanol, cleared in xylene and cover-slipped in permanent mounting medium. Images were taken with a Sony DSC-W 30 Cyber-shot digital camera (Sony, Tokyo, Japan). Subsequently, positive stained epithelial and stromal cells were visualized under a light microscope. The quantitative assessment of COX-2 and iNOS protein expression was conducted by counting 10 non-continuous sections per eye and a total of 20 eyes per group. The levels of expression of the proteins were expressed as percentage of the total cells counted.

**Statistical analysis**

Statistical analyses were carried out using one-way analysis of variance (ANOVA) to compare the experimental groups followed by Bonferroni's test for post hoc comparisons using SPSS for Windows (version 17). Results are presented as mean ± standard deviation. p < 0.05 was considered statistically significant.
RESULTS

Body weight gain and clinical observations

The body weight gain, disease activity index (DAI), colon length and weight, colon mass index and colonic weight/length ratio of control rats and those exposed to PCA alone, DSS alone, DSS plus PCA are presented in table 1. There was no mortality of animals in the present study. The DAI evidenced by the incidence of secretions and excretions (e.g. diarrhoea) and blood was significantly increased in rats exposed to DSS alone. Moreover, there was marked reduction in body weight gain and colon length (Figure 1B) in rats exposed to DSS alone when compared with the control. However, colon mass index and colon weight/length ratio were significantly increased in the rats exposed to DSS alone. The colon wet weight was not affected in all the treatment groups. There were no treatment related effects of PCA administration when compared with the control. Administration of PCA significantly decreased DSS-induced incidence of diarrhea and bleeding and restored the body weight gain, colon length, colon mass index and colon weight/length ratio in DSS-exposed rats.

Colonic and hepatic antioxidant status, nitric oxide concentration and myeloperoxidase activity

The effects of PCA on the colonic and hepatic antioxidant enzyme and MPO activities following induction of colitis by exposure to DSS are presented in table 2. The activities of SOD, CAT, GPx and GST in the colon and liver were significantly decreased in the rats treated with DSS alone when compared with the corresponding group of control animals. Also, administration of DSS alone significantly increased the colonic and hepatic MPO activity when compared with the
control. However, administration of PCA restored the activities of these enzymes, and maintained their normalcy in DSS-exposed rats except for hepatic GPx. Moreover, **Table 3** showed there was a significant decrease in the GSH level with marked elevation in the levels of NO, H₂O₂ and MDA in colons and livers of rats exposed to DSS alone when compared with the control group. Administration of PCA significantly increased GSH level and restored the levels of NO, H₂O₂ and MDA to normalcy in both colon and liver of DSS-treated rats. Administration of PCA alone did not affect the colonic and hepatic antioxidant status, NO concentration and MPO activity during this study.

**Markers of liver toxicity and pro-inflammatory cytokines levels**

**Table 4** shows the effects of treatments on the plasma markers of liver toxicity and inflammatory mediators. Administration of DSS alone resulted in a significant elevation in the markers of liver toxicity namely AST, ALT and ALP as well as in the pro-inflammatory cytokines namely TNF-α, IL-1β and IL-6 when compared with the control group. However, treatment with PCA significantly prevented the increases in these serum markers of liver damage and inflammatory mediators in DSS-treated rats. Neither the markers of liver toxicity nor the pro-inflammatory cytokines levels were affected following administration of PCA alone.

**Histological Observations**

Figures 2 and 3 are the representative photomicrographs of colon and liver sections of control and treated rats respectively. In **figure 2**, the histology of the colon sections of control and PCA alone-treated rats revealed normal structure having the usual epithelial architecture and adequate cryptal glandular epithelium (A and B). In contrast, treatment with DSS alone resulted in severe
sloughing off of the epithelium, edema, total absence of globet cells, marked necrosis of the crypts and cellular infiltration by mononuclear cells (C). The morphologic characteristics of the colons of rats treated with DSS plus PCA showed normal section with numerous goblet cells but with a few inflammatory cells present around the cryptal glands (D). In figure 3, the liver sections of control and PCA-treated rats revealed structurally and functionally normal hepatic cells (A and B). However, there are extensive infiltration of inflammatory cells and incidence of small-sized cytoplasmic vacuoles in the hepatocytes of rats treated with DSS alone (C). The morphologic characteristics of the livers of rats treated with DSS plus PCA appeared normal and comparable with control (D).

**Immunochemical analysis of COX-2 and iNOS**

The immunochemical analysis of COX-2 and iNOS expression in the colons of control and treated rats are presented in figures 4 and 5. The result revealed that the expression of COX-2 and iNOS in colons of rats exposed to DSS alone was more intense when compared with corresponding controls. However, the inhibitory effects of PCA treatment on the expression of COX-2 and iNOS were verified by the significant decrease in the expression of these proteins in the DSS plus PCA-treated rats. The arrowheads indicate where the intensity of immunostaining of COX-2 and iNOS appears increased compared with that in controls.
DISCUSSION

Biologically active dietary polyphenols have gained attention recently due to their involvement in the regulation of various cellular and molecular processes essential for the maintenance of body homeostasis. The beneficial health effects associated with the administration of PCA on DSS-induced colitis and hepatotoxicity was demonstrated in the present investigation. In the present study, rats exposed to DSS alone showed increased disease activity index (DAI) with decreased in the body weight gain. Further, the gross macroscopic pathology scores including the decrease in colon length, and the increase in colon mass index and colonic weight/length ratio were observed in the DSS treated rats. These clinical signs are well established in DSS-induced colitis and resemble the symptoms normally observed in human ulcerative colitis.\textsuperscript{3,16,29} Administration of PCA significantly reduced the DAI, increased the body weight gain and prevented the changes in colon length and mass index in the DSS-treated rats.

The levels of pro-inflammatory cytokines are influenced by several inflammation-related cells such as macrophages, neutrophils and leukocytes. Accumulation of leukocytes in the colon has been implicated to contribute to the mucosal tissue damage, breakdown of the mucosal barrier and subsequent inflammatory response.\textsuperscript{30} The present study showed significant increase in the plasma concentrations of IL-1β, IL-6 and TNF-α in rats treated with DSS alone. Our results are in agreement with previous observations from patients with ulcerative colitis as well as in experimental colitis.\textsuperscript{31,32} Pro-inflammatory cytokines could activate leukocytes and induce the expression of endothelial cell adhesion molecules which consequently increased the permeability of epithelial and vascular endothelial cell and colon damage in DSS-induced colitis.\textsuperscript{15} The
The present study confirmed the previously reported anti-inflammatory property of PCA evidenced by the suppression of IL-1β, IL-6 and TNF-α which are the key pro-inflammatory cytokines in DSS exposed rats.

The present study demonstrated a significant increase in colonic MPO activity and NO concentration in rats exposed to DSS alone. Nitric oxide is an extremely reactive endogenous substance commonly produced by activated macrophages and serves as a mediator for expressing cytotoxic activity. Overproduction of NO by inducible nitric oxide synthase (iNOS) in abnormal situations has been shown to induce inflammation. The increase in the colonic content of NO in DSS-exposed rats observed in the present study could lead to NO reaction with superoxide anion to form more poisonous nitrite anion and impair the colonic mucosa. Moreover, MPO is considered an index of inflammation damage and a biomarker of leukocytes infiltration. The increase in the colonic MPO activity observed in the present study indicates colonic inflammation damage resulting from the leukocyte infiltration in the DSS-treated rats. Similar increases in the MPO activity and NO concentration both in animals and humans with ulcerative colitis have been reported. The apparent reduction in DSS-mediated increase in MPO activity and NO concentration in rats treated with PCA indicates the ability of PCA to prevent neutrophil infiltration and inflammation in the colon.

The antioxidant defense system comprising the enzymatic enzymes SOD, CAT, GPx and GST as well as non-enzymatic GSH are very important because they are responsible for the direct removal of free radicals, consequently providing protection against oxidative damage in biological tissues. The present study showed that DSS treatment significantly decreased the
activities of SOD, CAT, GPx and GST in both colons and livers of experimental rats. Elevated level of ROS may inactivate and decrease the activities of these antioxidant enzymes, thus preventing their important role in protecting the cells from oxidative damage. Moreover, the marked decrease in the GSH was accompanied by significant elevation in the H$_2$O$_2$ and MDA levels in the colons and livers of DSS exposed rats. Our results corroborate the previous reports on the induction of oxidative stress in DSS-induced colitis. The depletion in the cellular antioxidants could induce oxidative stress and consequently lipid peroxidation in the colons and livers of rats exposed to DSS alone. However, administration of PCA enhanced the antioxidant status evident by the increase in the GSH level, antioxidant enzyme activities and a significant decrease in H$_2$O$_2$ and MDA levels in colons and livers of DSS-treated rats. The chemoprotective role of PCA could be attributed to its ability to scavenge free radicals which consequently relieved the inhibition of the antioxidant enzymes or increased their synthesis thus preventing oxidative damage in DSS-treated rats.

Light microscopic observation revealed morphological alterations such as severe sloughing off of the epithelium, edema, total absence of globet cells, marked necrosis of the crypts and cellular infiltration by mononuclear cells in rats exposed to DSS alone. Our observations are in agreement with earlier studies. However, colons of rats treated with PCA were comparable to the control in structure and function suggesting the chemoprotective ability of PCA in the colons of DSS-treated rats. Ulcerative colitis mediated hepatic damage was assessed by determining the activities of aminotransferases (ALT and AST) normally localized in periporal hepatocytes and ALP which resides in cells lining biliary duct of the liver. Elevated levels of ALT, AST and ALP in circulation indicate hepatic injury in the DSS-treated rats. The hepatic
damage was confirmed by the histological report. The restoration of all these liver function biomarkers following PCA treatment confirmed its chemoprotection against liver damage in DSS-treated rats. The biochemical evidence of the chemoprotection by PCA against ulcerative colitis mediated damage is well supported by the histological evaluation of liver.

Immunohistochemistry revealed an increased expression of COX-2 and iNOS in colons of rats exposed to DSS alone, thus corroborating the plasma biochemistry result of increased levels of pro-inflammatory cytokines. Cyclooxygenase-2, a rate limiting enzyme in the biosynthesis of prostaglandin from arachidonic acid, is well known to be involved in inflammatory processes.\textsuperscript{41} The increased expression of COX-2 and iNOS agreed well with an induction of inflammation in the colonic tissue of DSS-treated rats. Elevated expression of iNOS could result in excessive generation of cytotoxic NO molecules. The damaging effects of NO can be directly on the tissue or indirectly via production of poisonous nitrite anion.\textsuperscript{42} It is worthy of note that PCA treatment suppressed the COX-2 and iNOS expression thus, revealing its role in reducing NO-mediated oxidative stress and inflammatory response in the colonic tissue of DSS-treated rats. The mechanism by which PCA elicited the down-regulation of both COX-2 and iNOS expression remains to be determined. However, this chemoprotective effect of PCA could be attributed to its anti-oxidative and anti-inflammatory effects. The beneficial health effects of other plants-derived antioxidants and formulations in DSS-induced colitis in rodents are well reported.\textsuperscript{43,44}

In conclusion, the novel findings of the present study revealed the effective chemoprotective role of PCA in colitis and its associated hepatotoxicity. The beneficial effects of PCA are related to
the enhancement of antioxidant enzyme activities, inhibition of oxidative stress, decreased pro-inflammatory mediators and histological damages in DSS-treated rats.

Acknowledgement
This work was supported by the Multidisciplinary Research Grants under the Staff Training and Research Capacity Building Programme of the John D. and Catherine T. MacArthur Foundation Grant (USA) endowment from the University of Ibadan, Nigeria awarded to Professor E.O. Farombi.

Conflicts of interest
The authors declare there are no conflicts of interest.
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**Table 1:** Effects of PCA on body weight gain, disease activity index (DAI), colon length and weight, colon mass index and colonic weight/length ratio in DSS-treated rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PCA</th>
<th>DSS</th>
<th>PCA + DSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>BWG</td>
<td>8.65 ± 0.19</td>
<td>8.84 ± 0.16</td>
<td>1.85 ± 0.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.86 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DAI</td>
<td>0</td>
<td>0</td>
<td>7.03 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.42 ± 0.13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Colon length (cm)</td>
<td>21.68 ± 0.74</td>
<td>22.18 ± 0.92</td>
<td>16.45 ± 0.87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.28 ± 0.87&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Colon weight (g)</td>
<td>2.25 ± 0.02</td>
<td>2.27 ± 0.09</td>
<td>2.85 ± 0.06</td>
<td>2.24 ± 0.31</td>
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<tr>
<td>CMI</td>
<td>15.82 ± 1.92</td>
<td>15.03 ± 1.43</td>
<td>19.42 ± 1.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.62 ± 0.49&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Colonic wt/length</td>
<td>103.78 ± 3.65</td>
<td>102.34 ± 3.24</td>
<td>173.25 ± 3.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>110.45 ± 3.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

BWG, body weight gain; DAI, disease activity index; CMI, colon mass index. Values represent the mean ± SD of ten rats. <sup>a</sup>: Values differ significantly from control (p < 0.05). <sup>b</sup>: Values differ significantly from DSS group (p < 0.05).
Table 2: Effects of PCA on the activities of SOD, CAT, GST, GPx and MPO in colons and livers of DSS-treated rats.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PCA</th>
<th>DSS</th>
<th>PCA+ DSS</th>
</tr>
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<tr>
<td><strong>SOD</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Colon</td>
<td>1.15 ± 0.07</td>
<td>1.38 ± 0.50</td>
<td>0.66 ± 0.57^a</td>
<td>1.02 ± 0.50^b</td>
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<tr>
<td>Liver</td>
<td>1.10 ± 0.12</td>
<td>1.62 ± 0.16</td>
<td>0.81 ± 0.33^a</td>
<td>1.18 ± 0.09^b</td>
</tr>
<tr>
<td><strong>CAT</strong></td>
<td></td>
<td></td>
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<tr>
<td>Colon</td>
<td>7.98 ± 0.47</td>
<td>8.52 ± 0.62</td>
<td>5.53 ± 0.87^a</td>
<td>7.21 ± 0.98^b</td>
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<tr>
<td>Liver</td>
<td>14.94 ± 0.96</td>
<td>14.84 ± 0.92</td>
<td>10.23 ± 0.81^a</td>
<td>13.85 ± 0.79^b</td>
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<tr>
<td><strong>GST</strong></td>
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<tr>
<td>Colon</td>
<td>5.77 ± 0.51</td>
<td>5.71 ± 0.46</td>
<td>3.28 ± 0.59^a</td>
<td>5.58 ± 0.32^b</td>
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<tr>
<td>Liver</td>
<td>13.26 ± 1.87</td>
<td>15.57 ± 1.02</td>
<td>8.67 ± 4.40^a</td>
<td>13.14 ± 3.21^b</td>
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<tr>
<td><strong>GPx</strong></td>
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<td></td>
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<tr>
<td>Colon</td>
<td>12.36 ± 1.36</td>
<td>12.17 ± 1.62</td>
<td>8.52 ± 1.17^a</td>
<td>11.39 ± 1.18^b</td>
</tr>
<tr>
<td>Liver</td>
<td>10.74 ± 0.96</td>
<td>10.68 ± 0.92</td>
<td>7.98 ± 0.81^a</td>
<td>9.77 ± 0.98^a</td>
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<tr>
<td><strong>MPO</strong></td>
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<tr>
<td>Colon</td>
<td>0.26 ± 0.06</td>
<td>0.25 ± 0.08</td>
<td>0.96 ± 0.09^a</td>
<td>0.27 ± 0.08^b</td>
</tr>
<tr>
<td>Liver</td>
<td>0.54 ± 0.05</td>
<td>0.56 ± 0.06</td>
<td>1.26 ± 0.08^a</td>
<td>0.69 ± 0.07^b</td>
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</table>

SOD, Superoxide dismutase (Units/mg protein); CAT, Catalase (µmole H₂O₂ consumed/min/mg protein); GST, Glutathione S-transferase (µmole CDNB–GSH complex formed/min/mg protein), GPx, Glutathione peroxidase (Units/mg protein); MPO (Units/mg protein). Values represent mean ± SD of ten rats. ^a^: Values differ significantly from control (p < 0.05). ^b^: Values differ significantly from DSS group (p < 0.05).
Table 3: Effect of PCA on LPO, GSH, H$_2$O$_2$ and NO levels in colons and livers of DSS-treated rats.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PCA</th>
<th>DSS</th>
<th>PCA+ DSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPO</td>
<td>Colon</td>
<td>2.32 ± 0.96</td>
<td>2.81 ± 0.27</td>
<td>6.39 ± 0.25$^a$</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>1.80 ± 0.29</td>
<td>2.04 ± 0.28</td>
<td>4.64 ± 1.51$^a$</td>
</tr>
<tr>
<td>GSH</td>
<td>Colon</td>
<td>5.75 ± 0.43</td>
<td>6.19 ± 1.18</td>
<td>1.89 ± 0.31$^a$</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>14.75 ± 0.87</td>
<td>18.00 ± 5.66</td>
<td>8.47 ± 2.57$^a$</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Colon</td>
<td>13.25 ± 2.63</td>
<td>12.92 ± 2.18</td>
<td>21.69 ± 2.24$^a$</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>10.92 ± 3.54</td>
<td>9.75 ± 0.25</td>
<td>25.25 ± 6.19$^a$</td>
</tr>
<tr>
<td>NO</td>
<td>Colon</td>
<td>3.43 ± 0.56</td>
<td>3.36 ± 0.38</td>
<td>5.98 ± 0.39$^a$</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>2.22 ± 0.35</td>
<td>2.15 ± 0.59</td>
<td>4.87 ± 0.48$^a$</td>
</tr>
</tbody>
</table>

LPO, Lipid peroxidation (μmoleMDA formed/mg protein); GSH level (μmol/mg protein); H$_2$O$_2$ level (μmole H$_2$O$_2$ generated/mg protein); NO, Nitric oxide (Units/mg protein). Values represent mean ± SD of ten rats. $^a$: Values differ significantly from control (p < 0.05). $^b$: Values differ significantly from DSS group (p < 0.05).
Table 4: Effects of PCA on liver function indices and pro-inflammatory biomarkers

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PCA</th>
<th>DSS</th>
<th>PCA+ DSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST</td>
<td>13.50 ± 1.77</td>
<td>12.83 ± 1.13</td>
<td>39.42 ± 1.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.85 ± 1.42&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>ALT</td>
<td>10.80 ± 0.57</td>
<td>9.05 ± 0.07</td>
<td>24.40 ± 1.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.40 ± 0.85&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ALP</td>
<td>16.22 ± 0.96</td>
<td>15.76 ± 1.11</td>
<td>30.36 ± 2.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.48 ± 1.37&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-1β</td>
<td>47.31 ± 2.06</td>
<td>46.02 ± 2.08</td>
<td>72.85 ± 2.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.67 ± 2.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-6</td>
<td>22.54 ± 2.51</td>
<td>23.33 ± 2.27</td>
<td>48.78 ± 2.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.23 ± 2.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TNF-α</td>
<td>19.54 ± 2.08</td>
<td>20.1 ± 1.98</td>
<td>37.24 ± 2.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.55 ± 2.11&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

AST, Aspartate aminotransferase (U/L); ALT, Alanine aminotransferase (U/L); ALP, Alkaline phosphatase (U/L); IL-1β, Interleukin-1beta (pg/mL); IL-6, Interleukin-6 (pg/mL) and TNF-α, Tumor necrosis factor-alpha (pg/mL). Values represent mean ± SD of ten rats. <sup>a</sup>: Values differ significantly from control (p < 0.05). <sup>b</sup>: Values differ significantly from DSS group (p < 0.05).
Legend to Figures

**Figure 1**: (A) Chemical structure of Protocatechuic acid (PCA, 3,4-dihydroxybenzoic acid). (B) Image showing the representative whole colons excised from the various treatment groups.

**Figure 2**: Histological observation of the colon. (A) Control: Normal histological structure of mucosa. (B) PCA alone: No visible lesion; normal cryptal glandular epithelium. (C) DSS alone: There is marked necrosis of the crypts and sloughing off of the epithelium. (D) DSS plus PCA: Fairly normal section; there are a few inflammatory cells present around the cryptal glands; there are numerous goblet cells. X 250

**Figure 3**: Histological observation of the liver. (A) Control: No visible lesion. (B) PCA alone: No visible lesion. (C) DSS: There are widespread infiltration of inflammatory cells and small-sized cytoplasmic vacuoles in the hepatocytes. (D) DSS plus PCA: No visible lesion. X 250

**Figure 4**: Immunohistochemical staining showing the effect of PCA on COX-2 expression in the colon of DSS-treated rats. Control (A), PCA alone (B), DSS alone (C) and DSS plus PCA (D). Arrowheads indicate where the immunostaining intensity of COX-2 appears increased compared with that in controls. X 250. Effects of PCA on COX-2 expression in colons of DSS-treated rats (E) *: Values differ significantly from control (p < 0.05). **: Values differ significantly from DSS alone (p < 0.05).

**Figure 5**: Immunohistochemical staining showing the effect of PCA on iNOS expression in the colon of DSS-treated rats. Control (A), PCA alone (B), DSS alone (C) and DSS plus PCA (D). Arrowheads indicate where the immunostaining intensity of iNOS appears increased compared with that in controls. X 250. Effects of PCA on iNOS expression in colons of DSS-treated rats (E) *: Values differ significantly from control (p < 0.05). **: Values differ significantly from DSS alone (p < 0.05).
Figure 1
Figure 2:
Figure 3:
Figure 4:
Figure 5:

A: Image of tissue section. 
B: Image of tissue section. 
C: Image of tissue section with arrows indicating specific regions. 
D: Image of tissue section with arrow pointing to a specific region. 
E: Bar graph showing INOS positivity (%) for different conditions: Control, PCA alone, DSS, DSS + PCA.
Protocatechuic acid (PCA) ameliorates colitis and the associated hepatotoxicity through its intrinsic anti-inflammatory and anti-oxidative properties in dextran sulphate sodium-treated mice.