Protective effect of alcoholic extract of stem of *Entada pursaetha* in dextran sulphate sodium-induced colitis in mice

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Oxidative stress has been shown to play a critical role in the pathogenesis of ulcerative colitis (UC). *Entada pursaetha* has been demonstrated to have antioxidant and anti-inflammatory effects. In this study, we investigated the effects of stem of alcoholic extract of *E. pursaetha* (PSE) in dextran sodium sulfate (DSS)-induced colitis in mice. The protective effect of PSE was determined at three different doses of 30, 100 and 300 mg/kg body weight by oral gavage for 7 days. Morphological (colon length and colon weight/length ratio), clinical (disease activity index) and macroscopic (damage score) features were determined using standard criteria. Lipid peroxides (determined as malonaldehyde; MDA), enzymatic (superoxide dismutase; SOD and catalase; CAT) and non-enzymatic antioxidants (reduced glutathione; GSH), nitrate and nitrite (NOx) levels and myeloperoxidase (MPO) activity in colon tissues were determined. The DSS damaged the colonic tissue, increased MPO activity, lipid peroxidation and NOx levels, reduced the antioxidant enzymes and glutathione and lowered the body weight. PSE significantly reduced the inflammation of colon and reversed the increase in MPO activity induced by DSS. It also significantly increased the SOD and catalase activities and did not elicit any effect on depleted levels of GSH in the colonic tissue. In addition, PSE also significantly decreased colonic NOx and MDA levels compared to DSS-treated mice; reduced both infiltration of inflammatory cells and the mucosal damage in colon on histopathological examination. The results suggested the protective potential of PSE in DSS-induced colitis and this might be attributed to its anti-inflammatory and antioxidant activities.

**Keywords:** *Entada pursaetha*, Colitis, Oxidative stress, Antioxidant

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of intestine which mainly includes ulcerative colitis (UC) and Crohn’s disease (CD). UC and CD are characterized by mucosal inflammatory infiltrates, intestinal barrier function alteration and erosive loss of mucosa and submucosa. Because of extensive mucosal damage and massive infiltration of polymorphonuclear and mononuclear leukocytes, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced and released, resulting in potential oxidation and peroxidation of a large number of molecules\(^1\). When increased generation of highly toxic ROS exceeds the limited intestinal antioxidant defense system, it contributes to intestinal oxidative injury in UC patients\(^2,3\). The antioxidant compounds and free radical scavengers have been found to improve colitis in several colitis models in animals\(^4,5\).

The drugs used for the management of IBD include 5-aminosalicylic acid derivatives and systemic or local glucocorticoids which exert their beneficial effects through a combination of different mechanism\(^6,7\). However, these drugs are not devoid of potentially serious side effects, thus limiting their use\(^8,9\). As such, the development of new drug treatments that combine efficacy and safety is an important goal in IBD therapy. Natural products possessing several biological activities, mainly related to their ability to inhibit enzymes and/or their antioxidant properties have also been described to down-regulate the immune response\(^10\). Presently, the traditional medicines worldwide are being reevaluated by extensive research on different plants.

*Entada pursaetha* (syonyms: *E. rheedei, E. phaseoloides, E. scandens*) also called as Elephant...
Creeper is a very large gigantic woody climbing leguminous shrub. It grows in Central and Eastern Himalayas. Besides India, it is distributed in tropical forests of Africa, China, Philippines, Guam and Northern Australia. The bark is dark brown and rough. Mainly, its bark, seeds and vines are used for different medicinal purposes. In recent years, several activities of *E. pursaetha* have been described, such as antioxidant, anti-inflammatory, hepatoprotective, antipyretic, analgesic, in pain of loin, inflammatory glandular swelling and in liver diseases. There are evidences that natural compounds with antioxidant and anti-inflammatory properties have beneficial effects in UC. The extract of *E. pursaetha* has been described as an antioxidant with anti-inflammatory and immunomodulatory activities in several experimental settings. However, little is known about the protective effects of *E. pursaetha* against IBD, such as UC in humans or animal models. Therefore, in this study, we have investigated the protective effects of stem extract of *E. pursaetha* against dextran sulfate sodium (DSS)-induced colitis in mice, focusing on the anti-oxidant activity.

**Materials and Methods**

**Drugs and chemicals**

Dextran sulfate sodium (DSS; Molecular weight, 500 kDa) was purchased from SRL Chemicals Ltd., India. All the reagents for biochemical estimations were of analytical grade and procured from Sisco Research Laboratories, India. All other chemicals used were of analytical grade from Sigma-Aldrich, St. Louis (USA).

**Collection of plant material and extract preparation**

The stems of *Entada pursaetha* were obtained from the jungles of Bhawanipatna, District- Kalahandi, Odisha (India). The plant specimen was botanically authenticated by Dr. B N Pandey, Department of Botany, Bareilly College, Bareilly (India). A voucher specimen ID/EP-2010 was maintained in the Division of Pharmacology and Toxicology, Indian Veterinary Research Institute, Bareilly (India) for ready reference.

The stems collected from the fully mature plants were shade-dried, powdered and then extracted with 85% ethanol under reflux. The ethanolic extract of stem was concentrated to a semi-solid mass under reduced pressure and made free from any solvent. The alcoholic extract of *E. pursaetha* stem, hereafter referred as “PSE”, was suspended in 2% polysorbate 80 and used in different studies. The yield of the extract (PSE) was 8.4% with reference to dried starting material.

**Animals**

Colon-bred male mice (18-25 g) were obtained from the Laboratory Animal Resource Section of the Institute. The animals were acclimatized to the laboratory environment for 7 days prior to the start of the experiment. They were kept in polypropylene cages in a temperature-controlled room (22±2°C) with relative humidity of 30-70% and 12:12 dark: light cycle and maintained on a balanced ration and fresh drinking water was given *ad libitum*. Experimental protocols were approved by the Institute Animal Ethics Committee.

**Induction of colitis**

Colitis was induced in mice by chemical model with DSS. Mice were divided into 5 groups of 6 each. Group I received normal saline by oral gavage for 7 days and served as the naïve control. Mice of groups II to V were exposed to DSS (5%) via drinking water for 7 days for induction of colitis. Furthermore, group II concurrently received 2% polysorbate 80 aqueous solution and served as DSS-untreated control. Groups III, IV and V concurrently received PSE at 30, 100 and 300 mg/kg, respectively as suspension in 2% polysorbate 80 aqueous solution by oral gavage for 7 days. The doses of PSE (30, 100 and 300 mg/kg) were selected based on its anti-inflammatory activity carried out in our laboratory in rats (unpublished data). After 24 h of the last PSE administration, all animals were sacrificed under ether anesthesia for collection of colon for biochemical and histological assessment. Its length was determined with a scientific scale. Colon was cut open along the length and washed with normal saline solution to remove all the colon contents. It was block-dried, weighed and cut into separate portions.

**Assessment of DSS-induced colitis**

**Evaluation of disease activity index (DAI)**

Mice were examined for stool consistency (loose stool, diarrhea, tail soiling), occult and/or rectal bleeding (hemoccult positivity, gross bleeding) and body weight daily during the experimental period. The body weight loss was expressed as percentage weight loss (in 7 days) for each individual mouse.
and calculated by comparing body weight on day 1 with that taken at the 8th day before sacrificing the animals. These data were used to calculate DAI as described earlier\(^1\).

**Macroscopic assessment**
Tissues from distal colon were immediately examined under a stereomicroscope and changes were scored on a 0-5 scale according to the method originally described by Morris et al.\(^2\) for scoring of gross morphological (macroscopic) damage.

**Histopathological assessment of colitis**
Small pieces of colon, fixed in 10% neutral buffer formalin were processed for embedding in paraffin. Sections of 5-6 µm were cut, stained with haematoxylin and eosin (H&E)\(^21\) and examined for histopathological changes under the microscope (BX41TF, Olympus Microsystems Corp. Japan). Images were captured using an Olympus DP12CCD camera at original magnification of 100 (Olympus DP12 Microsystems Digital Imaging, Olympus, Japan). Histological evaluation was done in a blinded manner\(^22\).

**Preparation of colon homogenates**
Colon tissue (500 mg) was weighed and taken in 5 ml of ice-cold PBS (pH 7.4). Another 200 mg of sample was taken in 2 ml of 0.02 M EDTA solution for reduced glutathione (GSH) estimation. The colon homogenate (10%) prepared with IKA homogenizer (Germany) under ice-cold condition was centrifuged for 10 min at 3000 rpm. The supernatant was stored at -70°C until assayed for oxidative stress-related parameters. A double beam UV-VIS spectrophotometer (UV 5704 SS, ECIL, India) was used for recording the absorbance of the test samples.

**Assessment of oxidative stress**
Colonic level of lipid peroxidation was evaluated in terms of malondialdehyde (MDA) formed\(^23\). Colonic catalase (CAT) activity was assayed by the spectrophotometric method as described previously\(^24\). The superoxide dismutase (SOD) activity was estimated by the method of Madesh and Balasubramanium\(^25\). The GSH levels in colon tissue was determined by estimating free-SH groups using 5-5’ dithiobis 2-nitrobenzoic acid (DTNB) method\(^26\).

**Myeloperoxidase (MPO) activity assay**
The colonic MPO activity was determined by \(\alpha\)-dianisidine method, as described by Koike et al.\(^27\).

**Estimation of nitrate-nitrite in colon tissue**
Nitrate-nitrite levels, which are indicative of nitrosative stress indirectly indicate the level of nitric oxide (NO) production. Their levels were estimated by the method described by Sastry et al.\(^28\).

**Statistical analysis**
Data were expressed as mean ± SE. Statistical analysis of data was performed using Graphpad prism. Data were analyzed by ANOVA and means were compared with Tukey’s multiple comparison test. A minimum value of \(p < 0.05\) was considered statistically significant.

**Results**

**Effect of PSE on colon length and colon weight/length ratio**
Colon length was not significantly altered with any of the treatments. The average colon weight/length ratio in DSS-treated group was significantly higher than the naïve control group and this was not significantly altered with PSE treatment.

**Effect of PSE on DAI**
After three days of DSS, mice showed diarrhea and rectal bleeding which became more pronounced with the progress of DSS treatment suggesting development of colitis. DAI score of naïve control group was 0.00 ± 0.0. In DSS-treated group, there was >15% weight loss in animals. The stool consistency was loose or there was diarrhea. There was occult/gross rectal bleeding in animals. The DAI score (7.33 ± 0.55) of DSS-treated group was significantly higher than the score of naïve control group. Treatment with PSE at 30 mg/kg (6.00 ± 0.81) failed to produce any significant change in DAI score, whereas PSE in higher doses of 100 (4.33 ± 0.66) and 300 mg/kg (1.83 ± 0.40) significantly reduced DAI score (Table 1).

**Effect of PSE on colon macroscopic score**
The colon appeared flaccid and filled with liquid. Colon and rectum showed evidence of mucosal congestion and erosion, hyperemia and hemorrhagic ulceration in all the groups, except naïve control group. The macroscopic score for colon damage was significantly higher in DSS-treated group, compared to naïve control group. PSE failed to produce any significant decrease in DSS-mediated score. The macroscopic score of each group is illustrated in detail in Table 1.
Effect of PSE on colon histopathology

Negligible histological damage was observed in naïve control group. DSS-treated mice showed infiltration of neutrophils, macrophages, lymphocytes and eosinophils in the colonic mucosa and submucosa (Fig. 1). Loss of epithelium, crypt loss and depletion of goblet cells were seen (Fig. 1). The colons were graded for their histology score (Fig. 2). Animals receiving DSS had significantly higher histology score than naïve control animals. PSE reduced both infiltration of inflammatory cells and the mucosal damage, resulting in a significant reduction of histopathology score at 300 mg/kg.

Effect on MPO activity

The colon inflammation was quantitatively assayed by assessment of its MPO activity. Increased MPO activity was observed in DSS-treated group. PSE at 100 and 300 mg/kg suppressed MPO activity significantly in the colonic tissue (Fig. 3).

Effect on oxidative stress

In the DSS-treated mice, the level of MDA was significantly (74.23 ± 5.94 nM of MDA/g of tissue)

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**Table 1—Effect of *Entada pursaetha* stem extract (PSE) on colon disease activity index (DAI) score and macroscopic damage score in DSS-induced colitis in mice (n = 6)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>DAI score (Mean ± SE)</th>
<th>Macroscopic damage score (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive control</td>
<td>-</td>
<td>0.00 ± 0.00</td>
<td>0.16 ± 0.16</td>
</tr>
<tr>
<td>DSS</td>
<td>-</td>
<td>7.33 ± 0.55†††</td>
<td>3.33 ± 0.49†††</td>
</tr>
<tr>
<td>PSE</td>
<td>30</td>
<td>6.00 ± 0.81</td>
<td>2.83 ± 0.60</td>
</tr>
<tr>
<td>PSE</td>
<td>100</td>
<td>4.33 ± 0.66**</td>
<td>2.33 ± 0.49</td>
</tr>
<tr>
<td>PSE</td>
<td>300</td>
<td>1.83 ± 0.40***</td>
<td>1.50 ± 0.42</td>
</tr>
</tbody>
</table>

†††p<0.001 in comparison to naïve control; **p<0.01; *** p<0.001 in comparison to DSS-untreated control in Tukey’s multiple comparison post-hoc test.
increased, compared to the naive control (16.72 ± 1.72 nM of MDA/g of tissue) group. PSE at 100 and 300 mg/kg caused significant reduction in MDA levels (44.67 ± 2.84; 27.49 ± 1.32 nM of MDA/g of tissue), as compared to DSS-treated mice (Table 2). As shown in Table 2, compared to naive control group, the activities of SOD (16.22 ± 0.50 Units/mg protein), CAT (98.45 ± 5.65 mM of H$_2$O$_2$ consumed/min/mg protein) and GSH (2.19 ± 0.15 mM of GSH/g of tissue) decreased in DSS-treated mice. PSE elevated the activities of SOD at 100 (23.77 ± 0.56 Units/mg protein) and 300 (26.91 ± 1.24 Units/mg protein) mg/kg and of CAT at 300 mg/kg (144.15 ± 7.93 mM of H$_2$O$_2$ consumed/min/mg protein), but did not significantly alter the DSS-mediated GSH depletion.

**Effect on nitrate-nitrite levels**

DSS produced a significant elevation in the colonic content of total nitrate-nitrite, compared to naive control animals. PSE (30, 100 and 300 mg/kg) resulted in a significant decrease in colonic total nitrate-nitrite content, compared to DSS-treated mice (Fig. 4).

![Fig. 4—Effect of PSE on nitrate-nitrite levels in DSS-induced colitis in mice [n =6, †††p<0.001 in comparison to naive control; **p<0.01; ***p<0.001 in comparison to DSS-ununtreated control in Tukey’s multiple comparison post-hoc test]](image)

**Discussion**

We evaluated the protective effect of PSE against DSS-induced colitis in mice. This model exhibits symptoms comparable to those of human UC$^{29}$. DSS-induced colitis has been shown to be a good model for IBD. It produces significant colonic pathological changes in mice$^{10}$ and rats$^{11}$. Studies have also shown that agents effective clinically are also effective in this model$^{32,33}$. Our findings demonstrated that PSE treatment significantly suppressed DSS-induced colitis in mice by improving their body weight and stool consistency, as well as by decreasing intestinal bleeding, infiltration of leukocytes and mucosal damage. Further, PSE significantly inhibited DAI, histopathology score (Fig. 2) and MPO activity (Fig. 3). Though, we did not determine the LD$_{50}$ of PSE in this study, however, the oral LD$_{50}$ of crude ethanolic extract of *E. phaseoloides* in mice is found to be very high (27.17 g/kg body wt) in another study$^{34}$, suggesting that the extract is safe on long-term administration.

To our knowledge, this is the first report on the ability of PSE to inhibit DSS-induced experimental colitis. Our findings also suggested the anti-inflammatory effects of this extract as observed in other models of inflammation$^{13}$. One of the main pathological features of IBD is the infiltration of polymorphonuclear neutrophils and mononuclear cells into colonic tissues. Neutrophils enter the injured mucosa during acute gastrointestinal inflammation, leading to the overproduction of oxygen free radicals$^{1,35}$. Neutrophil infiltration into inflamed tissue plays a crucial role in destroying foreign antigens and in breaking down and rebuilding injured tissue. One measure of neutrophil infiltration is colonic mucosal MPO activity, which correlates well with the severity of the lesions in acute DSS-induced colitis$^{35-37}$. In our study, PSE treatment at 100 and 300 mg/kg significantly lowered the increase in

**Table 2**—Effect of PSE on colon reduced glutathione (GSH), lipid peroxidation (LPO), Catalase (CAT) and superoxide dismutase (SOD) levels in DSS-induced colitis in mice (n = 6)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>GSH (mM of GSH/g of tissue)</th>
<th>LPO (nM of MDA/g of tissue)</th>
<th>CAT (H$_2$O$_2$ consumed/min/mg protein)</th>
<th>SOD (Units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>-</td>
<td>3.40 ± 0.27</td>
<td>16.72 ± 1.72</td>
<td>170.84 ± 8.44</td>
<td>33.57 ± 0.74</td>
</tr>
<tr>
<td>DSS</td>
<td>-</td>
<td>2.19 ± 0.15††</td>
<td>74.23 ± 5.94††</td>
<td>98.45 ± 5.65††</td>
<td>16.22 ± 0.50††</td>
</tr>
<tr>
<td>PSE 30</td>
<td>30</td>
<td>2.36 ± 0.29</td>
<td>61.86 ± 2.47</td>
<td>109.53 ± 6.28</td>
<td>19.16 ± 1.07</td>
</tr>
<tr>
<td>PSE 100</td>
<td>100</td>
<td>2.61 ± 0.17</td>
<td>44.67 ± 2.84***</td>
<td>125.11 ± 3.48</td>
<td>23.77 ± 0.56***</td>
</tr>
<tr>
<td>PSE 300</td>
<td>300</td>
<td>2.88 ± 0.21</td>
<td>27.49 ± 1.32***</td>
<td>144.15 ± 7.93***</td>
<td>26.91 ± 1.24***</td>
</tr>
</tbody>
</table>

†p<0.01; ††p<0.001 in comparison to naive control; ***p<0.001 in comparison to DSS untreated control in Tukey’s multiple comparison post hoc test.
colonic mucosal MPO activity caused by DSS. Moreover, there was a significant attenuation of inflammation by PSE, as evident in histological examination and colonic MPO activity, which might be attributed to the reduction in neutrophil infiltration.

Several experimental studies have demonstrated an increased formation of ROS/RNS, including superoxide, hydrogen peroxide, hypochlorous acid and peroxynitrite in colonic mucosa in animal models of IBD. Formation of these reactive species is often observed at the early stage of disease process and is correlated with disease severity and progression. The activity of MPO, which is also a major enzyme in the formation of ROS, leading to tissue damage, increased in this colitis model. Increased MDA as a marker of lipid peroxidation observed in association with higher MPO activity in this experimental colitis model again confirmed the role of free radicals in IBD. Oxidative stress and its consequent lipid peroxidation could aggravate free radical chain reactions, disrupt the integrity of the intestinal mucosal barrier, and activate inflammatory mediators. It has been shown that colonic CAT and GSH levels are decreased, while lipid peroxidation (MDA levels) is increased in experimental and clinical studies. CAT and GSH, as primary defense molecules could reduce the oxidative stress and activation of inflammatory mediators.

PSE acting as free radical scavenger could counteract the function of ROS and directly scavenge radical species. The alcoholic extract of the stems of *E. phaseoloides* radical species. The alcoholic extract of the stems of *E. phaseoloides* has displayed potent antioxidant activity, when assessed by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical-scavenging, reducing power, carotene-bleaching and superoxide radical-scavenging analyses. Thus, PSE may possess similar kind of free radical scavenging potential. Our results suggested that PSE inhibited oxidative stress induced by DSS and the beneficial effects might partly be due to the antioxidant capacity of the compounds present in PSE.

In case of SOD deficiency or increased superoxide (O2•−) production, O2•− reacts with NO to produce RNS, such as peroxynitrite (ONOO−), which can cause oxidative/nitrosative stress and functional disorders in the cellular membranes and intracellular proteins. On the other hand, SOD plays an important role in protecting cells from oxidative damage by converting O2•− into H2O2. CAT transforms produced H2O2 into H2O. Many studies have demonstrated that antioxidant agents are beneficial against chemical-induced colitis. For example, SOD administration is reported to suppress DSS-induced colitis by decreasing ROS level in the colon and a similar result is reported in trinitrobenzene sulfonic acid-induced colitis in rat. Another report has demonstrated that ROS scavenger edaravone suppresses DSS-induced colitis. Our results were similar to these studies, indicating PSE might suppress the DSS-induced colitis through enhancement of antioxidant functions in the colon. It has become increasingly clear that NO overproduction by iNOS is deleterious to intestinal function, thus contributing significantly to gastrointestinal immunopathology during the chronic inflammatory events that take place in IBD. The important role attributed to NO in these intestinal conditions prompted us to study, whether the beneficial effects of PSE on DSS-induced colitis could be related to an effect on colonic NO production. This probably results from the intense macrophage activation, which takes place as a consequence of the inflammatory insult. In fact, macrophages are important source of pro-inflammatory mediators such as NO and TNF-α, playing a key role in the pathophysiology of IBD.

Colonic oxidative stress is a hallmark of IBD and colonic nitrite levels serve as a sensitive marker of disease activity in colitis. In another study, DSS colitis is found to be associated with a marked elevation in colonic total nitrate-nitrite level and this is in consonance with the previous reports showing similar results in experimental colitis paradigms. Increase in total nitrate-nitrite levels also appeared to relate to our histological findings, as there was infiltration of macrophage cells. The enhanced NO release observed in colonic tissue is a marker of toxicity which was attenuated by PSE.

Phytochemical screening of the PSE in our previous study revealed the presence of triterpenes, saponins, tannins, flavonoids and alkaloids. Further, we also determined various phytoconstituents (%) in PSE and found that the concentration of saponin was highest, followed closely by total phenols; total concentration of tannins and flavonoids was almost similar and had been reported earlier by our laboratory. Wide ranges of phytoconstituents are responsible for anti-inflammatory and antioxidant activity including phenolics, alkaloids, tannins, saponins and terpenoids. Several mechanisms of action have been proposed to explain the
anti-inflammatory actions of phytoconstituents: (i) antioxidative and radical scavenging activities, (ii) modulation of cellular activities of inflammation-related cells (mast cells, macrophages, lymphocytes, and neutrophils), (iii) modulation of proinflammatory enzyme activities, such as phospholipase A2 (PLA2), cyclooxygenase (COX) and lipoxygenase (LOX) and the nitric oxide NO producing enzyme, NO nitric oxide synthase (NOS), (iv) modulation of the production of other proinflammatory molecules and (v) modulation of proinflammatory gene expression. These phytoconstituents have been found to be effective in various models of IBD in mice and rats and may explain anti-inflammatory and antioxidant actions of PSE in IBD in mice in this study.

In conclusion, the present study demonstrated a protective effect of PSE in mice model of DSS-induced colitis, which was associated with reduced inflammation and peroxidative damage and improved antioxidant status.

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