Silibinin ameliorates Dextran Sodium Salt induced colitis in mice and prevents overexpression of inflammatory genes in lipopolysaccharide activated human macrophages

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The present work was undertaken to evaluate the anti-inflammatory effect of a pure compound silibinin on Dextran Sulfate Sodium (DSS)-induced mouse model of colitis. The expression levels of inflammatory markers such as IL-8, 5-LOX, COX-2 and iNOS in LPS activated human THP-1 derived macrophages cells were also measured. Swiss albino mice were treated with 2% DSS in their drinking water for seven days followed by one day with RO water. Silibinin (100mg and 200 mg per body weight) was administered daily through oral gavage for seven days and subsequently they were sacrificed and colon tissue samples were collected. Silibinin significantly attenuated DSS induced Disease activity Index (DAI) scores by shortening of colon length and decreased tissue Myeloperoxidase (MPO) activity that manifested as weight loss, diarrhea, rectal bleeding, and resulted in infiltrations of immune cells. Histological examination indicated that silibinin suppressed edema, mucosal damage, and the loss of crypts induced by DSS. Expression of inflammatory genes was assayed in LPS activated THP-1 derived macrophages by treating with silibinin for 24 hours when total RNA was extracted. Silibinin administration also effectively and dose dependently prevented expression of inflammatory marker genes in LPS activated THP-1 derived macrophages. These results suggest that silibinin has an anti-inflammatory effect at colorectal site suggesting its probable therapeutic role in ameliorating inflammation during colitis.

Keywords: Silibinin; Anti-inflammatory effect; Dextran Sodium Salt; colitis; inflammatory markers; THP-1 cells

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Introduction

Ulcerative colitis (UC) and Crohn’s disease (CD) are chronic inflammatory disorders of the intestinal tract. They are summarized as inflammatory bowel disease (IBD). In UC, the inflammation occurs in the mucosa and mucosal ulceration can develop. UC is mainly localized in the rectum and spreads to proximal parts of the intestine to a different extent. In CD, any part of the gastrointestinal tract can be affected, however the main site of inflammation is the terminal ileum and inflammation can
occurs segmental and discontinuously. In contrast to UC, not only the mucosa is affected but also all layers of the intestinal wall and granuloma are formed in CD. The pathogenesis of both diseases is not yet fully elucidated [2]. Different factors such as genetics, immune dysregulation, and the microbial flora in the intestine and barrier dysfunction of intestinal epithelial cells may lead to the pathology of IBD [3, 4]. The pathogenesis of IBD is poorly understood because of the variability in clinical manifestations and complexity of the mechanisms of chronic inflammation. There is increasing evidence that IBD tissue injury involves many cell types present in the bowel wall. The epithelial cell has been traditionally considered an important component of IBD pathogenesis, especially in UC. Due to the variability, complexity, and chronicity of gut inflammation in IBD, intestinal epithelial cells (IEC) constantly adapt to the multiple events occurring in the mucosal microenvironment, a phenomenon that has been long recognized. This adaptation might cause IEC to undergo changes in growth and differentiation, metabolism, secretary pattern, immune function, and antigen expression [5].

Dextran sulfate sodium (DSS)-induced colitis is one of the most common models of chemically induced colitis in mice [6]. DSS administered orally to mice initiates acute inflammatory bowel disease that resembles human ulcerative colitis [7]. Epithelial cell toxicity, increased intestinal permeability, and macrophage activation have been suggested as possible explanations for the deleterious effects of DSS although the mechanisms are not well understood. Intestinal microflora also plays a role in the development of the disease; however the mechanisms by which microflora influence mucosal immune responses are also unclear. During DSS treatment, the disease is characterized by colonic epithelial cell lesions and acute inflammation with infiltration of neutrophils and macrophages present within damaged segments [8]. This can be followed after termination of DSS treatment by the development of chronic colitis characterized by large numbers of activated T cells near diseased segments. Regeneration of the eroded epithelium occurs over the course of several days to weeks after DSS exposure.

Silibinin is a polyphenolic flavonoid (flavanone; Fig. 1) isolated mainly from the fruits or seeds of milk thistle (Silybum marianum (L.) Gaertn), which belongs to family Compositae [9]. Milk thistle is being used from the ancient time in traditional European medicine.

Silibinin is the major pure bioactive compound in milk thistle extract with small amounts of other stereoisomers, such as isosilybin, dihydrosilybin, silydianin and silychristin. Silibinin is widely used as a hepatoprotective, anti-inflammatory and antifibrotics agent [10]. Silibinin is known to exert a hepatoprotective effect as an anti-inflammatory agent both in vivo and in vitro, conditions as this inhibits tumor necrosis factor alpha (TNF-α) production by macrophages and monocytes [11].

In the current study we evaluated whether in vivo silibinin treatment may exert a protective effect on the DSS induced colitis model of mice. Different inflammatory parameters such as disease activity index, myeloperoxidase activity, colon length, histological changes were checked in mice after DSS and DSS + silibinin treatment. In vitro effect was studied using LPS activated THP-1 derived macrophages cells. Various inflammatory markers such as 5-LOX, COX-2, IL-8 and iNOS were checked by Real Time PCR after silibinin and LPS treatment in THP-1 differentiated macrophages.

**Materials and methods**

**Animals**

Swiss albino male mice, 6-8 weeks old weighing 30-36g were used. Mice were kept on 12/12 h light/dark cycle. The mice were fed standard chow formula and RO water ad libitum and allowed to acclimatize for one week. All the protocols were approved by the Institutional Animal Ethics Committee of Jawaharlal Nehru University, New Delhi, India.

**Experimental colitis**

Experimental colitis was induced in mice by administering 2% DSS (w/v) solution in RO water over the experimental period. Silibinin (Sigma Aldrich, India) was administered orally in concentration of 100 mg/kg/day and 200 mg/kg/day. Mice were randomly divided into four groups each containing 5 mice: (1) nontreated group, (2) only DSS treated group (3) DSS and 100mg/kg/day silibinin treated group (4) DSS and 200mg/kg/day silibinin treated group.

**Disease activity Index (DAI)**

During the period of 7 days, daily routine clinical evaluations included: weight loss, Hemoccult test or rectal bleeding and stool consistency. DAI was calculated by scoring changes as described by Cooper [8] and shown in Table 1.

DAI score was graded on a scale of 0 to 4. DAI was
Colon length

At the end of the experiment, mice were killed by cervical dislocation, and laparotomy was performed. Colon was excised, freed of adherent adipose tissue. Subsequent to washing in ice-cold 0.9% saline solution, the colon was excised, freed of adherent adipose tissue. Subsequent to washing in ice-cold 0.9% saline solution, the colon was placed on filter papers to measure their length. Colon length was taken from caecum to anus.

Myeloperoxidase assay (MPO assay)

Approximately 100mg tissues from colon region were snap frozen in liquid nitrogen and homogenized in 1ml of hexadecyltrimethyl ammonium bromide (HTAB) dissolved in potassium phosphate buffer. Tissue particulate was discarded by centrifugation (5000rpm, 2 min) and supernatant was collected. 10μl of supernatant was taken in triplicate in 96 wells plate. For blank 10μl of HTAB buffer was taken in triplicate. 200μl of Potassium phosphate buffer (pH 6.0) containing 0.5mM o-dianisidinedihydrochloride (MP Biochemicals Inc., Osaka, Japan) and 0.05% hydrogen peroxide was added. Optical density was measured immediately at 450nm at room temperature (25°C). One more reading was taken between 30 and 60 seconds. Average of two readings (ΔA0-30 and ΔA30-60) was taken and MPO was calculated using formula:

\[
MPO (U/gm of tissue) = \frac{\text{Average of } \Delta \text{A}0-30 \text{ and } \Delta \text{A}30-60}{(\text{Time} \times (\text{MPO constant}) \times (\text{tissue weight in gm}))}
\]

MPO constant is 1.13 x 10⁻².

Histopathology

For histopathology analysis, samples from the mid-part of the colon were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned (5µm), stained with haematoxylin and eosin (H and E), and examined at x20 magnification.

Animal Cell culture

The human monocytic cell line THP-1 (NCCS, Pune, India) was maintained in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Invitrogen), 584mg/L L-glutamine, 4500 mg/L glucose, penicillin (50 units/ml) and streptomycin (50 µg/ml) in a fully humidified atmosphere with 5% CO₂ at 37°C.

In vitro treatment of silibinin in LPS-activated Phorbol 12-myristate 13-acetate (PMA) differentiated human THP-1 macrophages

To check the anti-inflammatory effect of silibinin in vitro, THP-1 derived macrophages were used. Human THP-1 cells (5 x 10⁵ to 10⁶ per ml) were differentiated in macrophages by inducing cells with 50 ng PMA for 48 hours in 6 well plates. Then THP-1 macrophages were pre-incubated for 24 h with silibinin (25μM, 50μM, 100μM and 150μM) and further incubated for 6h with 1 µg/ml of LPS in 6-well plates. Total RNA was extracted and expression of inflammatory marker genes was analyzed by RT-PCR.

RNA extraction and Real Time PCR for IL-8, iNOS, COX-2 and 5-LOX

Total RNA was extracted from cells using Trizol reagent (Sigma Aldrich, India). The concentration of RNA was adjusted to 1μg µL⁻¹ with RNase free distilled water. Reverse transcription of total RNA was performed by means of the Revert Aid First Strand cDNA Synthesis kit (Fermentas, St. Leon Rot, Germany) using 1µg of total RNA per sample in a final volume of 20µl. The quality of cDNA was checked by normal PCR reactions. Reverse-transcribed products were utilized for Real time PCR in 7500 Real time PCR system using SYBR green universal PCR master mixture from Applied Biosystems as per the instructions of the supplier. Prior to each quantitative real-time PCR, the cDNA was diluted appropriately. For all experiments, RNA of untreated cells were isolated (control)
at all the time points and the GAPDH normalized values (ΔCT) of the stimulated HT 29 cells were expressed relative to the normalized values of the respective control cells (ΔΔCT). We carried out an independent experiment to show that GAPDH was not regulated by the applied mediators used in our study. The CT values of all genes ranged from 20 to 30. To quantify gene expression, the comparative threshold cycle method for relative quantification (2^−ΔΔCT = n fold) was used. The effects of stimulators were checked by screening at different time points where the effect on expression had the greatest impact.

Statistical analysis

All data were analyzed using the paired student’s t-test. These data are presented as mean±SEM, data were analyzed by one way ANOVA test. A level of p<0.05 was considered significant. These exercises were done with graphpad calculator available on www.graphpad.com/quickcalc by GraphPad software Inc.

Results

Silibinin decreased DAI scores in mice model of colitis

Administration of 2% DSS was found to be associated with significant clinical changes, which included weight loss, diarrhea, and the appearance of occult fecal blood. Silibinin drug was administered orally. Treatment with silibinin, an herbal product (100 and 200 mg/kg/day for 7 days) suppressed the pathological conditions in a dose-dependent fashion and significantly reduced intestinal inflammation by simultaneous improvement in DAI score (Fig 2).

Silibinin prevented shortening of colon

To determine whether silibinin has a beneficial effect on DSS-induced colon shortening, we measured and compared the colon lengths of untreated control mice, mice with DSS-induced colitis, and in mice co-treated with silibinin (100 and 200 mg/kg/day) + DSS. Significant shortening of colon length was observed in mice with DSS-induced colitis as compared to untreated. Oral administration of silibinin reduced shortening of colon length at both the concentrations 100mg/kg/day and 200mg/kg/day (Fig 3).

Silibinin prevented increase in MPO level

As previously discussed MPO level is considered as a specific biomarker of inflammation. We found that an elevated MPO level correlated with the development of colonic inflammation during colitis and that the administration of silibinin (100 and 200 mg/kg/day) significantly suppressed MPO accumulation in the colonic tissues of mice with DSS induced colitis (Fig 4). There was significant increase in MPO activity in only DSS treated mice as compared to control (untreated mice). Further when compared with only DSS treated mice, 100mg/kg/day (p<0.05) and 200mg/kg/day silibinin treated mice (p<0.001) showed decreased MPO activity.

Changes in histological parameters before and after silibinin treatment

Pathological examinations of colons were carried out by haematoxylin and eosin (H&E) staining and representative results are shown in Fig 5. Tissue sections from colon region showed distortion of epithelium in the crypt region with simultaneous infiltration of inflammatory cells as expected in colitis induced mice compared to control mice. However, when the mice were co-treated with silibinin, the level of distortion reduced significantly (Fig 5 c, d). Interestingly, crypts structures were rather well-preserved and inflammatory reactions were significantly lower in tissue samples from mice treated in combination with DSS and silibinin (100 and 200 mg/kg/day) than only DSS treated mice.

Changes in the expression of inflammatory marker genes in LPS activated human THP-1 derived macrophages

Expression of four inflammatory marker genes 5-LOX, COX-2, IL-8 and iNOS (Primers are given in Table 2) was measured in THP-1 derived macrophages analyzed after LPS+ silibinin treatment by RT-PCR (Fig 6). All the four
Evidence indicated increased colon length both at very high dose. This indicated that silibinin prevented accumulation of inflammatory cells at the site of inflammation. Decrease in MPO may be the result of some unknown intracellular signaling pathway that needs to be worked out further.

Histological changes such as crypts destruction, mucosal thickening and epithelial layer erosion are hallmark of colitis. Our observation further revealed that silibinin was also effective in preventing histopathological damages such as crypt destruction, mucosal thickening and epithelial layer damage.

To get an insight into the signaling pathway, we further studied in vitro anti-inflammatory effect of silibinin by treating the human THP-1 differentiated macrophages with silibinin and then activated with LPS. The expression of four inflammatory markers IL-8, COX-2, 5-LOX and iNOS were measured by RT-PCR. IL-8, a pro-inflammatory cytokine is known to increase during IBD and acts as a powerful neutrophil chemo attractant. Thus increase or decrease of IL-8 showed corresponding increase or decrease in infiltration of neutrophils at inflamed site. In our study we found silibinin at all concentrations decreased the expression of IL-8. This indicated that silibinin indirectly reduced neutrophils accumulation by preventing IL-8 over expression.

The cyclooxygenase (COX) is a key enzyme used in the conversion of arachidonic acid to prostaglandins. COX-2 gets rapidly up regulated by growth factors and cytokines and thus play a role in inflammation. Increase in COX-2 expression leads to increased formation of prostaglandins. Accumulation of prostaglandins finally leads to inflammation. In our study silibinin decreased COX-2 expression in a dose dependent manner. Therefore we hypothesized that silibinin decreased COX-2 expression probably by preventing IL-8 over expression during inflammation.

The important role of the enzyme 5-lipoxygenase (5-LOX) is the production of leukotrienes (LTs) during the inflammatory diseases. IBD is also caused as a result of increased generation of LTs in the inflamed mucosa. In our study we found silibinin decreased 5-LOX expression. Therefore silibinin may also prevent inflammation due to its 5-LOX inhibitory property. Further study is required to confirm the exact pathway silibinin adopts that result in 5-LOX inhibition.

Nitric oxide (NO) is considered to be a pleiotropic free radical messenger molecule. Large body of evidence indicate that the inducible form of the NO synthase enzyme (iNOS) that is responsible for high-output production of NO from l-arginine is up-regulated in various forms of

Figure 5. H & E stained cross section of colon of mice after silibinin treatment. a- control mice showed normal histological structures. b- Treatment with DSS caused the loss of epithelial layer, mucosal thinking and loss of crypts and infiltration of neutrophils. c and d- DSS + silibinin treatment prevent the loss of epithelium, crypt structure and less accumulation of neutrophils compared to only DSS treated mice. Images were taken at 200x.
mucosal inflammation. Consistent with this, multiple detection strategies have demonstrated that iNOS expression, enzymatic activity, and NO production are increased in human inflammatory bowel disease tissues. There is also evidence that the level of iNOS-derived NO correlates well with the disease activity in ulcerative colitis [18]. We observed decreased iNOS gene expression in all the concentrations of silibinin used.

Thus we can conclude that silibinin is an effective compound in preventing DSS induced colitis. Our *in vitro* study also showed that silibinin acts as an anti-

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**Table 2. Genes with Primer sequences and product size**

<table>
<thead>
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<th>Gene</th>
<th>Product</th>
<th>forward &amp; reverse primer</th>
<th>Size</th>
</tr>
</thead>
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<tr>
<td>iNOS</td>
<td>Forward 5'-TGTGCCACCTCCAGTCCAG-3'</td>
<td>Reverse 5'-GACCTGCAAGTTAAAATCCC-3'</td>
<td>239bp</td>
</tr>
<tr>
<td>IL-8</td>
<td>Forward 5'-CTGATTTCGAGCTCTGTTG-3'</td>
<td>Reverse 5'-CATCAGAAAAAGCTTAAATATT-3'</td>
<td>177bp</td>
</tr>
<tr>
<td>COX-2</td>
<td>Forward 5'-ACAGCCAGAGCCCTAGACA-3'</td>
<td>Reverse 5'-AGGATTGGCTGTATGCTGACG-3'</td>
<td>122bp</td>
</tr>
<tr>
<td>5-LOX</td>
<td>Forward 5'-AATATCGATGGATGAGTGGGA-3'</td>
<td>Reverse 5'-ATGAAGCGGTTGATGAAACAGGT-3'</td>
<td>156bp</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Foward 5'-GCTCCTCCACTGCACAGTC-3'</td>
<td>Reverse 5'-GCAAAATATCCACTTACCAG-3'</td>
<td>189bp</td>
</tr>
</tbody>
</table>

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**Figure 6. Effect on inflammatory genes in THP-1 derived macrophages.** Expression of inflammatory marker genes 5-LOX, COX2, IL-8 and iNOS after LPS and silibinin treatment in human macrophages. a) 5-LOX, b) COX2 expression, c) IL-8 gene expression and d) iNOS gene expression in LPS+silibinin treated cells. Data represents mean (n=3/group) ±SEM, Student t test was used to calculate significance level (a: significant change from control group, b: significant change from DSS treated group).
inflammatory agent by preventing over expression of inflammatory genes such as cytokine IL-8, and enzyme 5-LOX and iNOS. Decrease in IL-8 leads to suppression of COX-2 and neutrophils accumulation at the site of inflammation. It will be interesting to check the effect at the protein level.

**Conflict of interest**

The authors have declared that no competing interests exist.

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**References**