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Modulatory effects of sesamol in dinitrochlorobenzene-induced inflammatory bowel disorder in albino rats

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Abstract:
Background: Inflammatory bowel disease (IBD) is a chronic inflammatory condition of gastrointestinal tract of immune, genetic and environmental origin. In the present study, we examined the effect of sesamol (SES), the main anti-oxidative constituent of *Sesamum indicum* (sesame seed) Linn. in the dinitrochlorobenzene (DNCB)-induced model for IBD in rats.
Methods: The groups were divided into normal control, DNCB control, SES and sulfasalazine (SS). On day 24, the rats were killed, colon removed and the macroscopic, biochemical and histopathological evaluations were performed.
Results: The levels of MPO, TBARS and nitrite increased significantly ($p < 0.05$) in the DNCB group, whereas reduced significantly in the SES, SS treated groups. Serum nitrite levels were found to be insignificant between the different groups. IL-6 and TNF-α levels were significantly high in the DNCB group.
Conclusions: We conclude the mucosal protective effect of SES on colon due to its potent antioxidant actions. Further investigation is required in a chronic model of different rodent strain for its role involved in the cytokine pathway.

Key words:
sesamol, dinitrochlorobenzene, myeloperoxidase, TBARS, TNF-α, interleukin-6

Abbreviations: CD – Crohn's disease, DNCB – dinitrochlorobenzene, IBD – inflammatory bowel disease, IL – interleukin, MPO – myeloperoxidase, ROS – reactive oxygen species, SES – sesamol, SS – sulfasalazine, TBARS – thiobarbituric acid reactive species, TNF – tumor necrosis factor, UC – ulcerative colitis

Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of gastrointestinal tract which is usually presented as Crohn's disease (CD) or ulcerative colitis (UC). It is characterized by mucosal tissue damage which is initiated by dysregulation in inflammatory system. The etiological factors for IBD include smoking [7, 33] diet [1], toothpaste [19], intestinal permeability [14], colonic mucus [27], familial [8, 34] and genetic [3, 31, 37] factors. The pathways to mucosal inflammation include two categories with respect to effector T cells (T_E) and regulatory T cells (T_R). If T_R cells decrease abnormally or T_E (T_H1 and T_H2) cells increase, it leads to inflammation [5]. On the other hand, reactive oxygen species (ROS) also plays an important role in the tissue injury in the progression of IBD. Neutrophil derived H_2O_2 [23] was considered to be the major cause of tissue damage

than the weak oxidant superoxide ion [18]. Recent findings have proven that increased production of ROS is one of the etiological factors that lead to the development of IBD [26, 35]. Therefore, we hypothesized that antioxidants may suppress the excessive ROS in the inflamed intestine of IBD patients. Hence, we selected a compound sesamol which has potent anti-oxidant [13], anti-inflammatory [6] and free radical scavenging activity [25]. Sesamol promoted dermal healing [32], particularly epithelialization; hence it might promote the mucosal healing by suitably modulating the inflammatory cytokines in IBD. Another study demonstrated that SES *in vivo* strongly inhibited lipid peroxidation. This contributes to the anti-oxidative properties of sesame lignans and reduces the susceptibility to some forms of oxidative stress [16]. For the induction of IBD, we selected DNCB (2,4-dinitrochlorobenzene) which acts as a hapten and induces colitis by activating the T cells when bound to the protein of the colon [29]. In the present study, we estimated the levels of myeloperoxidase (MPO), thiobarbituric acid reactive species (TBARS), tissue nitrite, interleukin(IL)-6, tumor necrosis factor(TNF)-α and histological findings in the colon.

Materials and Methods

Chemicals

DNCB, 2-thiobarbituric acid, and trichloroacetic acid were obtained from Hi-Media Laboratories Pvt. Ltd., India. Sesamol (3,4-methylenedioxyphenol), *o*-dianisidine dihydrochloride, and Griess reagent were obtained from Sigma-Aldrich, St. Louis, MO, USA. IL-6 and TNF-α kit were obtained from Invitrogen, USA. Total Protein kit was obtained from Thermo Fisher, Pierce, USA. Analar grade chemicals were used.

Animals

Healthy inbred male albino rats of Wistar strain (220–250 g) were used. The rats were kept in air-conditioned room maintained at a temperature of 23 ± 2°C with a 12 h light/dark cycle. The animals had free access to standard pellet diet and water *ad libitum*. The experiments were approved by Institutional Animal Ethical Committee (IAEC) (vide # IAEC/ KMC/88/ 2011–2012) and were carried according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Intra-rectal administration of DNCB was carried out under diethyl ether anesthesia.

Induction of colitis and treatments

The protocol and dosing strategies were taken from the previous study [36]. Twenty four animals were divided into 4 groups of 6 animals each as follows: normal control, DNCB control, sesamol treated (SES) and sulfasalazine treated (SS). On day 1, hair on the nape of the rats was removed by applying 10% sodium sulfide (Na_2S). After 2 h, 300 µl of DNCB in acetone (20 g/l) was applied on the same spot with a microtiter pipette. From days 2–14, spraying of DNCB onto the nape of rats was continued. On day 14, animals were fasted overnight and on day 15, 250 µl of DNCB (0.1% DNCB in 50% ethanol) solution was instilled into the rectum of the rat by using an *iv* cannula of 16G around 6–8 cm from the anus. From day 16–23, control received 0.3% CMC (*po*), SES group received sesamol (100 mg/kg, *po*) and SS group received sulfasalazine (100 mg/kg, *po*). Finally, on day 24, the animals were killed by cervical dislocation and dissected open to remove the colon. The entire colon starting from cecum was taken and placed on a slab for measuring the length and weight. Around 6–7 cm of proximal part of colon was taken for biochemical estimation which includes nitrite, TBARS and MPO by placing them in physiological buffer pH 7.4 until the homogenization of the samples were carried out. A small part of proximal colon was taken for histopathological study and stored in 10% formalin for histological studies. Before killing the animals, blood was collected from retro-orbital sinus under light anesthesia, serum and plasma were separated individually from each rat and the samples were estimated for nitrite levels.

Homogenization of samples

The samples were homogenized in an ice container at a concentration of 10% (w/v) in 1.15% (w/v) solution of potassium chloride by using a glass homogenizer. After this, the homogenized samples were centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was pipetted out with a microtiter pipette and separated into aliquots for individual biochemical estimations.

Assay of colonic MPO

MPO is an enzyme found in the intracellular granules of neutrophils which can be utilized as an indirect measure of the neutrophil content of the tissue sample [20]. The entire estimation was carried out in a 96-well plate and the readings were taken on an ELISA plate reader (BioTek, USA.) at 490 nm. A sample of 50 µl was taken in duplicate. To this, 250 µl of ODA-H_2O_2 was added which comprises of 680.45 mg of potassium dihydrogen orthophosphate in 100 ml of distilled water and the pH was adjusted to 6.0. ODA solution includes 0.167 mg of ODA in 1 ml of phosphate buffer of pH 6.0. Finally, ODA-H_2O_2 was prepared by adding 1 ml of 30% H_2O_2 in 1 ml of ODA solution. After addition, the reading was noted at 5 and 15 min. After this, 4 M H_2SO_4 was added to stop the reaction and once again the reading was noted. The concentrations of MPO at subsequent time intervals were determined from standard plot which uses horse radish peroxidase as standard. Note: H_2O_2 & ODA solutions are light sensitive, so wrapped in aluminum foil. The entire experiment was done under dark conditions especially addition of ODA-H_2O_2 solution.

Assay of lipid peroxides in colonic homogenates

Malonaldialdehyde (MDA), which is formed by the breakdown of polyunsaturated fatty acids (PUFA), serves as an index for determining the extent of peroxidation reaction [21]. To 250 µl of colonic homogenate, 250 µl of TBA (2-thiobarbituric acid)-TCA (trichloroacetic acid) reagent was added. The reagent comprised of 15% (w/v) of TCA, 0.375% (w/v) of TBA, 15 mg of butylated hydroxytoluene (BHT) and 200 µl of 0.25 M hydrochloric acid. The solution was kept in a sonicator for 0.5 h and gently heated on a magnetic stirrer for about 1 h to assist the dissolution of TBA. After addition, these samples were heated on a water bath for 40 min at 80°C. After heating, the samples were centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was transferred to a 96-well plate for measuring the absorbance at 432 nm. The concentrations of MDA were determined by constructing a standard plot by 1,1,3,3-tetramethoxypropane.

Nitrite assay

During inflammation, macrophages and neutrophilic granulocytes of intestinal mucous membrane are activated and release large amounts of toxic NO, which would damage the intestinal mucous membrane or even react with superoxide anion ($O_2^{\bullet-}$) and produce more active oxidizing substance called oxidized nitrous acid ($OONO^-$) [22]. The cell membranes and organelles contain proteins and lipids which are oxidized by these oxidizing species and destruct the tissue in terms of free radical chain reaction so that the integrity of mucus membranes as a barrier is destroyed [10]. In this assay, 100 µl of sample (serum or colonic tissue homogenate) was taken in a 96 well plate. To this, 100 µl of Griess reagent was added, incubated for 10 min and then absorbance was measured at 540 nm. The concentrations were calculated with standard plot by using sulfanilamide as standard.

Tissue IL-6 and TNF-α estimations

The IL-6 and TNF-α levels were found out according to the protocol given by Invitrogen.

Statistics

The results are expressed as mean ± SEM. Statistical significance was calculated by analysis of variance (ANOVA) followed by *post-hoc* Tukey's multiple comparison test using Graph pad Prism version 5.0; $p < 0.05$ was considered to be significant.

Results

Body weight

From Figure 1, it can be seen that there is a significant decrease in the mean of end weight as percentage of initial weight in DNCB, SES groups when compared to control at $p < 0.05$, which was found to be 93.83 ± 1.29 and 96.82 ± 3.50%, respectively. The SS treatment group did not show any significant reduction in the body weight.

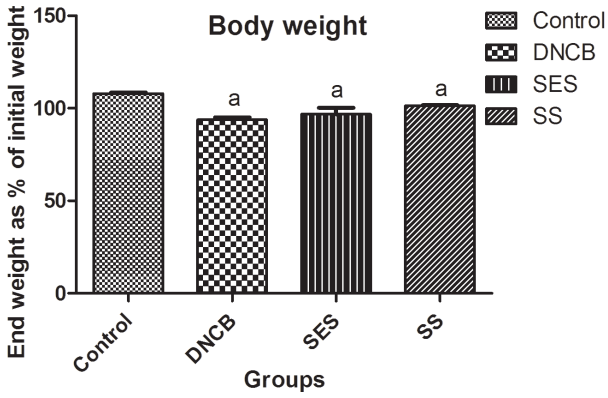

Fig. 1. Effects of different treatments on mean of end body weight as percentage of initial body weight. [a] $p < 0.05$ when compared with control

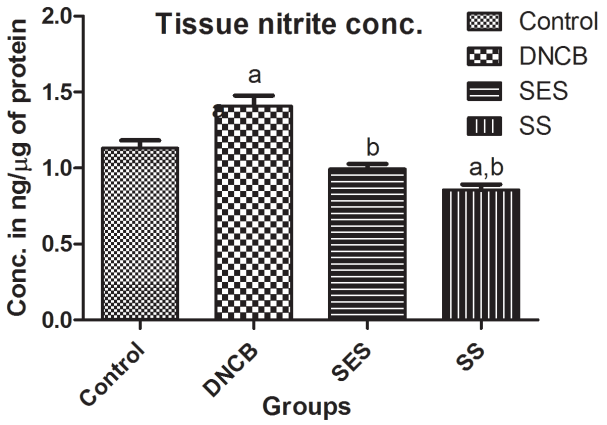

Fig. 4. Effect of different treatments on the levels of nitrite in colon tissue homogenate. [a] $p < 0.05$ when compared with control; [b] $p < 0.05$ when compared with DNCB

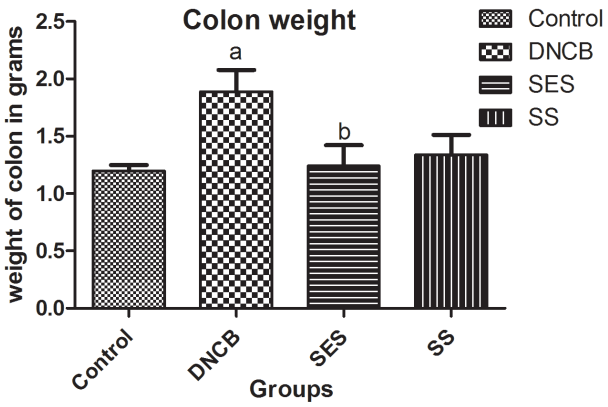

Fig. 2. Effects of different treatments on colon weight. [a] $p < 0.05$ when compared with control; [b] $p < 0.05$ when compared with DNCB

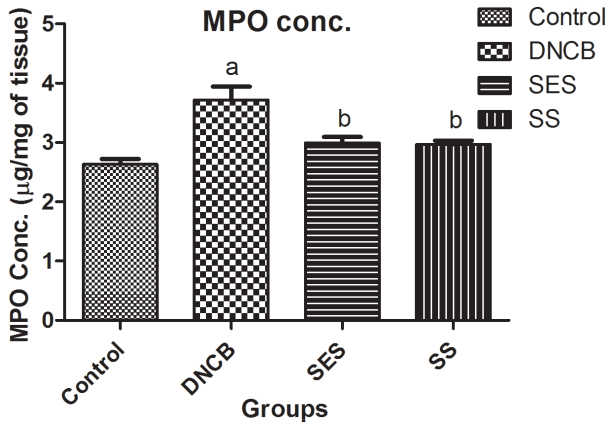

Fig. 3. Effect of different treatments on the concentration of MPO at 15 min. [a] $p < 0.05$ when compared with control; [b] $p < 0.05$ when compared with DNCB

Colon weight

Figure 2 shows a significant increase in the weight of colon with respect to control in only DNCB group at $p < 0.05$, which was found to be 1.88 ± 0.18 g but when compared to DNCB group there was a significant decrease in the weight of the colon in the SES treated group at $p < 0.05$, which was found to be 1.24 ± 0.17 g.

MPO estimation

It is evident from Figure 3, that when compared to control group there was a significant rise in levels of MPO at $p < 0.05$ in the DNCB group, which was found to be 3.71 ± 0.22 µg/mg of tissue. There was a significant decrease in the levels of MPO in drug treated groups (SES and SS) compared to DNCB group at $p < 0.05$, which was found to be 2.99 ± 0.09 and 2.96 ± 0.06 µg/mg of tissue, respectively. In between the standard and test drug treatment groups, no significant difference was observed.

Tissue nitrite estimation

Figure 4 depicts a significant increase in the levels of nitrite in the DNCB group at $p < 0.05$. Compared to DNCB, both SES and SS treated groups showed a significant decrease in the levels of tissue nitrite. This was found to be 0.99 ± 0.03 and 0.85 ± 0.03 ng/µg, respectively.

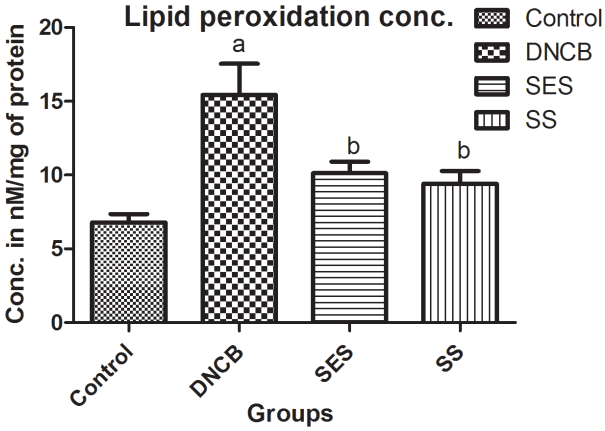

Fig. 5. Effect of different treatments on the levels of malondialdehyde (MDA). [a] $p < 0.05$ when compared with control; [b] $p < 0.05$ when compared with DNCB

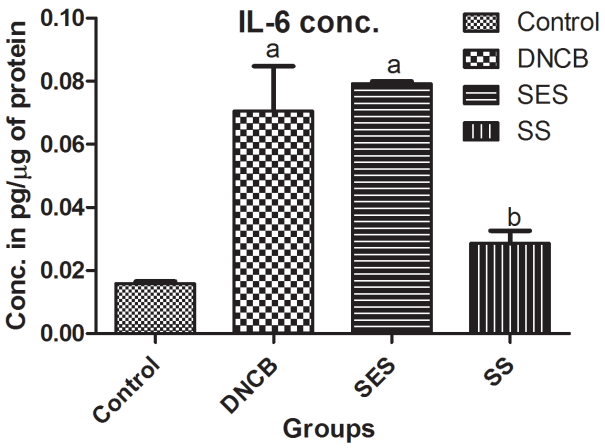

Fig. 6. Effect of different treatments on the levels of IL-6 in colon tissue homogenate. [a] $p < 0.05$ when compared with control; [b] $p < 0.05$ when compared with DNCB

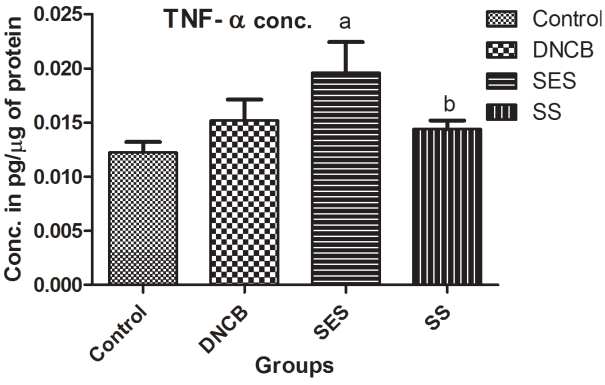

Fig. 7. Effect of different treatments on the levels of TNF-α in colon tissue homogenate. [a] $p < 0.05$ when compared with control; [b] $p < 0.05$ when compared with DNCB

TBARS estimation

Figure 5 exhibits a significant rise in the levels of MDA in the DNCB group at $p < 0.05$, which is found to be 0.565 ± 0.062 nM/mg of protein. When compared to DNCB group, there was a significant decrease in the levels of MDA in SES and SS treatment group, which was found to be 0.352 ± 0.028 and 0.327 ± 0.043 nM/mg of protein, respectively, at $p < 0.05$.

Estimation of IL-6 in the colonic tissue homogenates

There was a significant rise in the levels of IL-6 in all the groups except the SS treatment group when compared with the control at $p < 0.05$, which were found to be 0.070 ± 0.0141 pg/µg (DNCB only), 0.079 ± 0.0006 pg/µg (SES) and 0.0286 ± 0.004 pg/µg (SS), respectively. When compared with DNCB group, none of the groups showed significant decrease in the levels except the standard SS treatment group as shown in Figure 6.

Estimation of TNF-α in the colonic tissue homogenates

From Figure 7, it can be seen that there was a significant rise in the levels of TNF-α in the SES treated group at $p < 0.05$ but the DNCB control and SS treated groups did not show any significant rise in the levels of TNF-α, which was found to be 0.0151 ± 0.00193 pg/µg (DNCB only), 0.019 ± 0.00285 pg/µg (SES treated) and 0.014 ± 0.00079 pg/µg (SS treated). When compared to DNCB group, only SS treated showed a significant decrease in the levels of TNF-α at $p < 0.05$.

Estimation of serum nitrite

Results are expressed as concentration of nitrite in ng and also percentage decrease with respect to control in the serum. As shown in Figure 8, when compared to control, none of the groups showed a significant decrease in the levels of nitrite in the serum.

Histopathological studies

The normal histology of the colon is shown in Figure 9A. In Figure 9B, colonic ulceration and congestion were clearly seen in the DNCB treatment. Crypt ab-

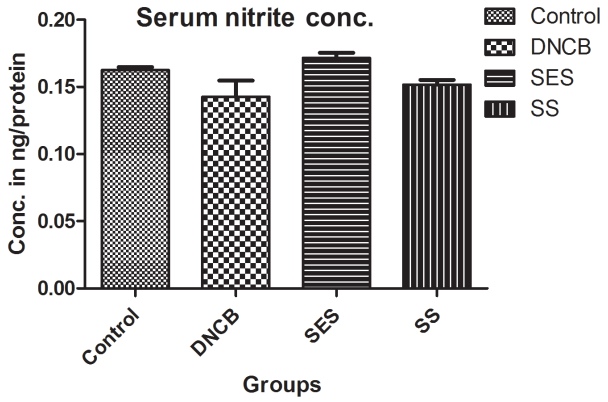

Fig. 8. Effect of different treatments on the levels of serum nitrite. The data represent the mean ± SEM of three readings

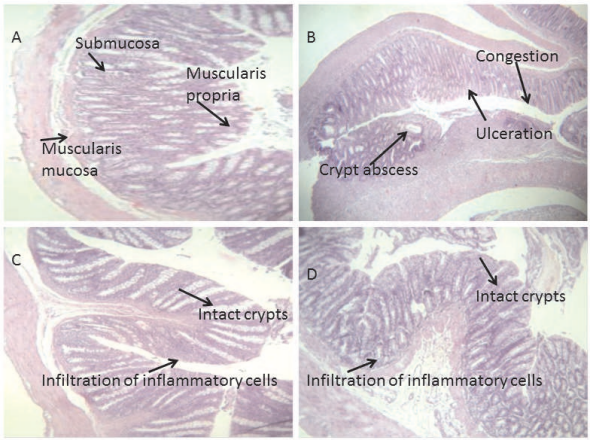

Fig. 9. Histo-pathological features of the colonic tissue. (**A**) Control; (**B**) DNCB; (**C**) Sesamol; (**D**) Sulfasalazine treated groups

scesses were observed in the muscularis propria (Fig. 9B). Sesamol and sulfasalazine treated groups were similar to control depicting normal crypts and a few inflammatory cell infiltration (Figs. 9C and 9D respectively).

Discussion

IBD comprises of chronic, uncontrolled inflammation of intestinal mucosa [24], which can be diagnosed by the presence/absence of architectural distortion (e.g., transmural or superficial patchy granulomatous infiltration) and/or acute inflammatory cells. The inflammatory responses, once activated, in patients of IBD were unable to subside when compared to the normal individuals [11]. When healthy individuals are exposed to a potential pathogen, the intestine becomes inflamed and once the pathogen is eradicated from the gut, it returns to a state of tolerance. On the contrary, in IBD patients, inflammation is not down-regulated; but the intestinal mucosal immune system remains chronically activated and inflamed.

The ultimate goal of understanding IBD is to provide relief to the patients by regulating the environmental/genetic/inflammatory factors. Of the mentioned factors, modulation of inflammatory mediators is of primary interest. In the progression of the disease, ROS play an important role [23]. Hence in this study, we have used SES, which has a potent anti-oxidant activity [32], free radical scavenging activity [25] but its role in various chemically induced models of IBD has not been found out.

DNCB by acting as a hapten, produces a secondary immune response when placed on the skin and on continuous application accelerates the immune response due to alteration of colonic antigens which finally leads to tissue necrosis and edema [30].

In this study, we have used DNCB model for the development of IBD in albino strain of Wistar rats. In this model, the status of inflammation is well linked with that of the human inflammatory bowel symptoms; also resembles clinical pathology of colon [10]. In the present study, the antioxidant effect of SES was assessed on the gross parameters (body weight, colon weight and colon length) and biochemical parameters (MPO, lipid peroxidation, nitrite, IL-6 and TNF-α).

Weight loss is mainly due to abdominal pain and anorexia [4]. There was significant reduction in the body weight in the DNCB and SES treatment group when compared to control.

MPO activity gives a quantitative measure of disease severity and a method of evaluating drug action in animal models of intestinal inflammation [20]. In our experiment, MPO activity in the inflamed colon was determined. The drug SES was able to produce a reduction in the MPO activity, which can be considered as a manifestation of the anti-inflammatory activities of the test compound in the DNCB model.

In the present study, DNCB group showed a significant rise in the lipid peroxides that was in parallel with the depleted reduced glutathione (GSH) content (results not shown), which is indicative of oxidative stress. The test drug was able to combat oxidative

stress by reducing the colonic tissue contents of lipid peroxides. The undetectable levels of GSH were supported by earlier reports [12].

From our findings, we did not observe any significant difference in the serum nitrite of the various treatment groups when compared to control, unlike previous reports suggesting a reduction in the serum nitrite of the treated group reported that in the inactive colitis patients there is no significant difference in the levels of serum nitrite [17], whereas those patients who are active showed a remarkable change in the levels of serum nitrite [28], which indicates that the inflammatory mediators are limited to colonic tissue rather than systemic, which we confirmed by estimating the levels of WBC in the blood plasma. On the other hand, there was a significant decline in the tissue nitrite levels of the treated groups when compared to control speculating it to be confined to the colon [9], rather than being systemic. The decreased levels of nitrite in the treatment group indicated the mucosal protective effect of SES on colon, which was further confirmed with histopathology findings.

DNCB group significantly increased the IL-6 cytokine levels after intra-colonical challenge. The drug SES was unable to reduce the raised cytokine levels indicating that the drugs may act through ROS pathway. The bioavailability of SES given orally was found to be 35.5 ± 8.5% [15] and lower amount of the drug is absorbed in the jejunum and ileum before reaching the colon; because of this it can act as an effective antioxidant [32] able to suppress MPO [23], TBARS and NO rather than the cytokine reduction. The SS treatment group showed a significant decrease in the levels of IL-6 indicating that it is a potent anti-inflammatory drug.

Our experiments showed that the pro-inflammatory cytokine TNF-α production was enhanced in colonic mucosa after DNCB instillation. The drug SES increased the levels of TNF-α significantly when compared to control, which indicates that they may induce the production of other cytokines including adhesion molecules, arachidonic acid metabolites, and activation of immune and non-immune cells [9].

The goal of IBD therapy is to induce and maintain remission. The current treatment paradigm involves a step-up approach, moving to aggressive, powerful therapies only when milder therapies with fewer potential side effects fail or when patients declare themselves to have an aggressive disease [2]. We conclude that the drug SES showed comparable activity with SS. Further investigation is required to evaluate the effect of this drug on the cytokine pathway in a chronic model of IBD using different strains of rodents.

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