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EFFECT OF *NIGELLA SATIVA* IN EXPERIMENTALLY INDUCED INFLAMMATORY BOWEL DISEASE IN RATS

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ABSTRACT

Inflammatory bowel disease (IBD) (ulcerative colitis and Crohn's disease) is an idiopathic, chronic inflammatory condition, which affects the gastrointestinal tract.The present study was carried out to evaluate the effect of ethanolic extract of *Nigella sativa* in experimentally induced inflammatory bowel disease (IBD) in rats. Colitis was induced by a single intra-colonic application of 20 mg 2,4,6-trinitrobenzene sulfonic acid (TNBS) dissolved in 35% ethanol into the descending colon. Rats were divided into six groups. Animals were treated with vehicle (ethanol), TNBS dissolved in 35% ethanol, Ethanolic extract of *Nigella sativa* seeds (ENS) 100, 200 and 400 mg/kg body weight p.o. and sulfasalazine(SSZ) 360 mg/kg body weight p.o. for 14 days. After completion of 14 days of treatment, animals were sacrificed and the following parameters were assessed morphological score, histopathology and biochemical parameters like myeloperoxidase (MPO), malondialdehyde (MDA), reduced glutathione (GSH), catalase (CAT), superoxide dismutase (SOD) activity and serum nitrate levels. *Nigella sativa* provided protection against TNBS-induced colonic damage. There was significant protection with ENS 200 and 400 mg/kg body weight compared to control ($P < 0.001$). Morphological and histological score were significantly reduced in all the treated groups ($P<0.001$). All parameters were altered in ulcerated rats, and improved in animals receiving ENS, an effect that was comparable to that of the standard sulfasalazine, especially at the highest dose level. Results indicate efficacy of ENS against TNBS induced experimental colitis in rats.

Key words: IBD, ENS, TNBS, MPO, MDA, Anti-oxidant enzyme.

INTRODUCTION

Inflammatory bowel disease (IBD) (ulcerative colitis and Crohn's disease) is an idiopathic, chronic inflammatory condition, which affects the gastrointestinal tract. The etiology of IBD remains obscure, although it is believed that an alteration in the intestinal immune system contributes to the inflammation that occurs. IBD is characterized by an up-regulation in the synthesis and release of different pro inflammatory mediators, including reactive oxygen and nitrogen metabolites, eicosanoids, platelet-activating factor and cytokines. All of these mediators contribute to the pathogenic cascade that initiates and perpetuates the inflammatory response of the gut. The available treatment choices have major limits owing to associated side effects and toxicity. As a result, there is high prevalence of complementary and alternative medicines for treating IBD[1-3].

The *Nigella sativa* belongs to family Ranunculacae, commonly known as black cumin seed known to have a great medicinal importance possessing many medicinal properties particularly in Greco-Arab, Unani-Tibb and Ayurvedic system of medicine. The seeds have claimed to have several traditional medicinal properties [4]. Recently, the seeds have been reported to exhibit many pharmacological effects

including immunomodulator[5], anticancer[6], anti-diabetic[7], anti-hypertensive[8], hepatoprotective [9], anti-oxidant[10], anti-bacterial activity[11], anti-helminthes [12], and anti-inflammatory[12]. This study is designed to evaluate different dose effect Extract of *Nigella sativa* on TNBS induced IBD in rats.

MATERIALS AND METHODS

Chemicals

Ethanolic extract of *Nigella sativa* (ENS) (NASC-0076**)** a gift sample obtained from Madhur Pharma, Bangalore, India, TNBS (2,4,6-trinitrobenzene sulphonic acids was purchased from Sigma Chemical, USA), Sulfasalazine (Wallace Pharmaceuticals, Mumbai, India). All other chemicals were of analytical grade.

The dried ethanolic extract was suspended in distill water using 1% Tween 80, used for pharmacological screening.

EXPERIMENTAL ANIMALS

Experimental animals

Adult Swiss albino mice (20- 25g) and Wistar rats (150 -200g) of either sex were used for the study. The mice and rats were fed with standard pellet (Parnava Agro industries Ltd. Sangali, India) and water *ad libitum*. The animals were maintained under standard 12-hr light / dark cycle throughout the study. The study protocol was approved by IAEC. (No.CPCSEA/IAEC/PC-01/346)

Acute toxicity study [14]

The study was performed according to the acute toxic classic method (as per CPCSEA/OECD guidelines). Swiss albino mice were used for acute toxicity study. The animals were kept fasting for overnight providing only water, after which the test drug extract dissolved in Water was administered orally at the dose of 800 mg/kg and observed for 14 days. Animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24 h (with special attention during the first 4 h) and daily thereafter for a period of 14 days. Once daily cage side observations included changes in skin and fur, eyes and mucous membrane (nasal) and also respiratory rate, circulatory(heart rate and blood pressure), autonomic (salivation, lacrimation, perspiration, piloerection urinary incontinence, and defecation) and central nervous system (ptosis, drowsiness, gait, tremors and convulsion). The toxicity study carried out as per the guidelines of AOT- 421 using albino mice. The extracts were found to be safe till 800mg/kg. Hence we selected 100 mg, 200 mg and 400 mg/kg dose (P.O) for pharmacological screening.

Induction of colitis

Colitis was induced by a single intra-colonic application of 20 mg TNBS dissolved in 35% ethanol using a rubber catheter of diameter 2mm. The catheter was lubricated with glycerin and inserted rectally into the colon through anus such that the tip was 8cm inside from the anus. The total volume was expelled with additional air. Rats were observed for 2 weeks and sacrificed under anesthesia using anesthetic ether and by cervical dislocation for assessment of various parameters. During and after TNBS administration, the rats were kept in a head-down position until they recovered from the anesthesia for a few minutes to prevent leakage of the intracolonic instillation of TNBS, and they were returned to cages.

Groups

The animals were divided into six groups, each containing six rats.

Group I animals were administered phosphate buffer saline intra colonically. Group II were administered TNBS (20mg) dissolved in 0.25ml of 35% ethanol [13]. Group III, IV and V were administered TNBS (20mg) dissolved in 0.25ml of 35% ethanol + ENS 100mg/kg, 200mg/kg and 400mg/kg, p.o). Group VI were administered TNBS (20mg) dissolved in 0.25ml of 35% ethanol + Sulphasalazine [13] (SSZ) 360 mg/kg, p.o. After 14 days of treatment, animals were sacrificed and the following parameters were assessed, colon weight/ length ratio, morphological index, histopathology, myeloperoxidase activity, malondialdehyde activity, reduced glutathione, catalase and superoxide dismutase activity and serum nitrate levels.

Assessment of colitis: The colonic segments were placed on an ice-cold plate, cleaned of fat and mesentery and blotted on filter paper. The colon was weighed/cm length and expressed as mg/cm tissue length (CW/LR)[13].

Macroscopic assessment: The colon longitudinally opened and scored for macroscopically visible damage using magnifying lens according to the criteria, which take into account the extent as well as the severity of colonic damage [1].

Histopathological studies: The colonic specimens was taken from a region of the inflamed colon immediately adjacent to the gross macroscopic damage in the distal colon of each animal and was fixed in 4% buffered formaldehyde. Sections of tissue was cut (5µm), stained with hematoxylin and eosin (H and E) and evaluated by light microscopy for morphological changes [1]**.**

Biochemical parameters assessment: MPO activity, an indicator of polymorphonuclear leukocyte accumulation, was determined by the tetramethylbenzidine (TMB) method, MDA, GSH, catalase and superoxide SOD levels in the colonic tissue and serum nitrate levels were determined[14-15].

Statistical analysis: All data were expressed as mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using instat statistical software. Analysis of variance (ANOVA) followed by Tukey Multiple Comparison Test. A value of P< 0.05 was considered as the level of significance.

RESULTS

Administration of TNBS 20mg single dose, intra colonically showed significantly higher CW/LR value when compared to normal control rats. ENS treated rats showed CW/LR values significantly lower which was comparable to standard sulfasalazine treated group (Table 1). The morphology of colon of TNBS administered rats' revealed inflammatory response with presence of inflammatory changes in the mucosa. ENS and SSZ treated group showed MI (macroscopic index) significantly lower compared to TNBS group (Table 1). However, the histopathology of colon administered with TNBS showed mucosal ulceration, transmural inflammation, diffuse infiltration of inflammatory cells in the mucosa and submucosa. Histopathology of colon administered with ENS (100mg/kg, p.o) showed focal ulceration and inflammation extending to muscularis propria, whereas histopathology of colon administered with ENS (200mg/kg and 400mg/kg p.o) showed healing of intestinal wall with inflammation limited only to submucosa and mucosa which was comparable to standard SSZ (Figure 1-6).

The tissue Myeloperoxidase (MPO) and Malondialdehyde (MDA) levels are markers for inflammatory tissue damage and lipid peroxidation. In TNBS administered group there was significant higher MPO and MDA levels compared to normal control rats. The tissue MPO and MDA levels in rats treated with ENS 200mg & 400mg/kg p.o) showed significantly lower when compared to TNBS administered group. The tissue MPO and MDA levels in rats administered with SSZ showed significantly lower when compared to TNBS administered group (Table 1).

In TNBS administered group there was a significant decrease in colonic non-enzymatic GSH content and enzymatic CAT, SOD defense systems compared to normal group. GSH, CAT and SOD in rats treated with ENS 200mg & 400mg/kg p.o), standard SSZ showed significantly lower when compared to TNBS administered group (Table 1).

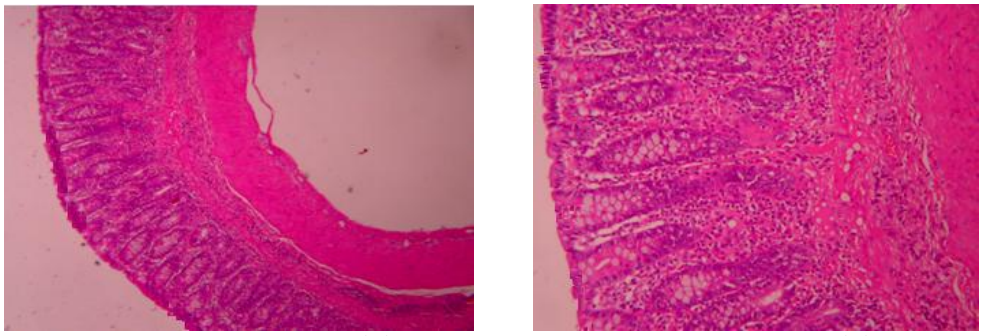
In addition, serum nitrate level was significantly increased in TNBS colitis group when compared to the normal group and significantly lower in ENS 200mg & 400mg/kg p.o and standard SSZ treated group compared to the TNBS administered group..

Table.1 Comparison of different parameters measured in TNBS induced IBD model in rats.

Parameters	Normal	TNBS	ENS (100mg/kg)	ENS (200mg/kg	ENS (400mg/kg)	SSZ (360mg/kg)
CW/LR (mg cm^{-1})	60±4.27	176±7.43[a]	167±10.28	114.5±5.24[c]	106±8.47[c]	93.5±10.2[c]
MI (0-10)	0.33±0.21	7±0.23[a]	6.5±0.42	1.62±0.21[c]	1.33±0.21[c]	1±0.25[c]
MPO (U/g tissue)	22.01±0.21	68.08±1.8[a]	64.07±1.58	43.70±1.65[b]	32.21±0.7[c]	24.8±0.11[c]
MDA(nmol/g wet tissue)	15.89±0.49	78.32±3.5[a]	71.34±3.3	40.13±2.03[b]	27.36±1.7[c]	22.32±1.0[c]
GSH (nmol/ g wet tissue)	1100±18.2	782±14.6 [a]	940±14.7	911±10.7 [b]	948±19.3 [c]	988±18.4 [c]
SOD (U/mg protein)	6.23±0.02	3.6±0.4[a]	4.01±0.2	4.41±0.8[b]	6.22±0.20[c]	8.25±0.3[c]
CAT (U/mg protein)	22.6±0.22	10.42 ±0.1[a]	14.8±0.2	14.05±0.25 [b]	17.2±0.3 [c]	20.8±0.3 [c]
Serum nitrate (µ mol/L)	16.48±1.38	48.66±2.2 [a]	34.81±1.26	25.05±2.04 [b]	18.35±1.33 [c]	17.06±1.23 [c]

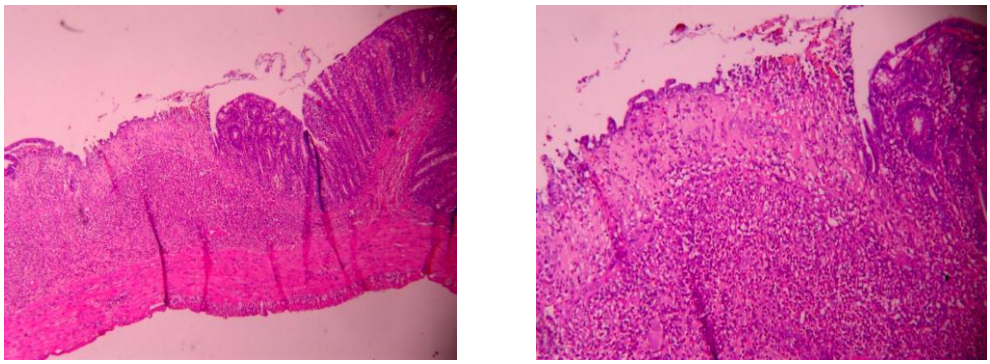
Score data were expressed as mean ± S.E.M, n=6. ; (ANOVA) followed by Tukey Multiple Comparison Test
[a]P<0.001 vs Normal group; [b]P<0.01, [c]P<0.001 vs TNBS group

Histological Sections Of Colonic Mucosa



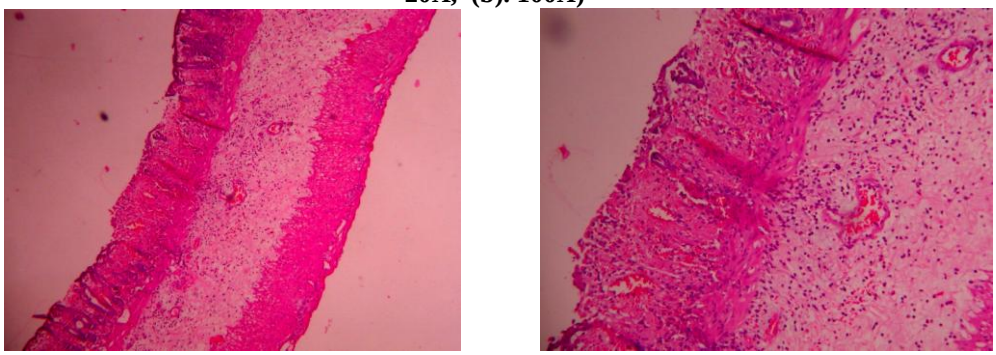
(A) (B)

Figure 1 Histological sections of colonic mucosa in normal group showing normal histology of rat colon with intact epithelial surface. (Magnification; (a). 20X, (b). 100X)



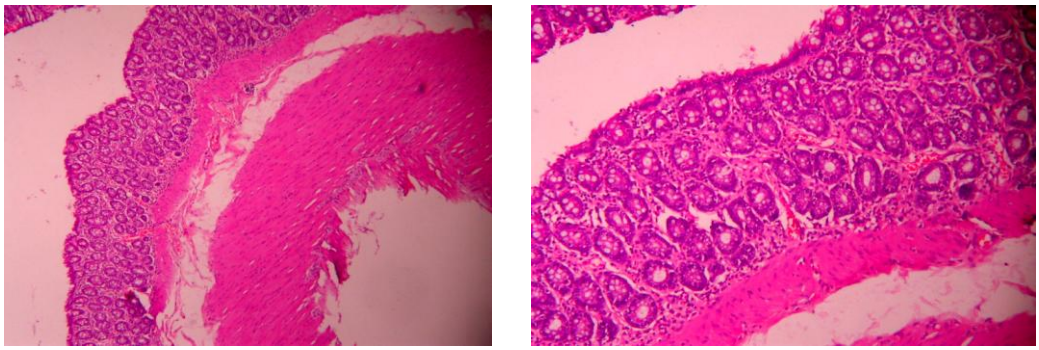
(A) (B)

Figure 2 Histopathoological sections of colonic mucosa in TNBS group showing mucosal ulceration and transmural inflammation most evident in the enlarged sub mucosa of rat colon. (Magnification; (a). 20X, (b). 100X)



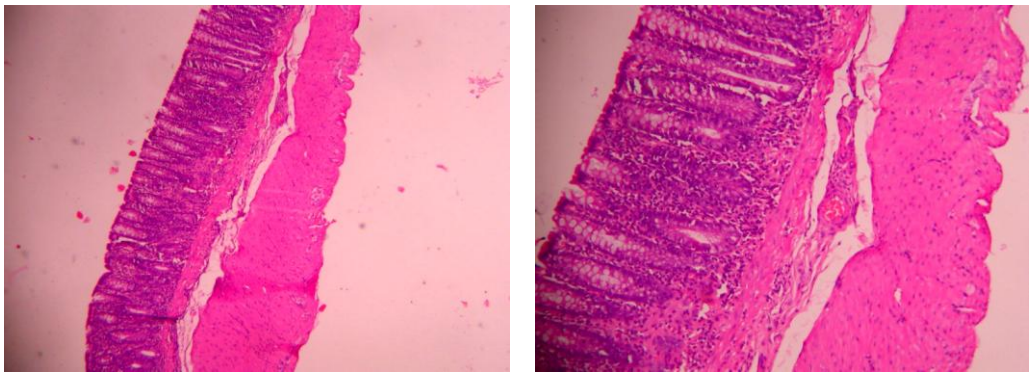
(A) (B)

Figure 3 Histopathoological sections of colonic mucosa in ENS 100mg/kg administered rat colon showing focal ulceration and inflammation with involvement of muscularis propria. (Magnification; (a). 20X, (b). 100X)



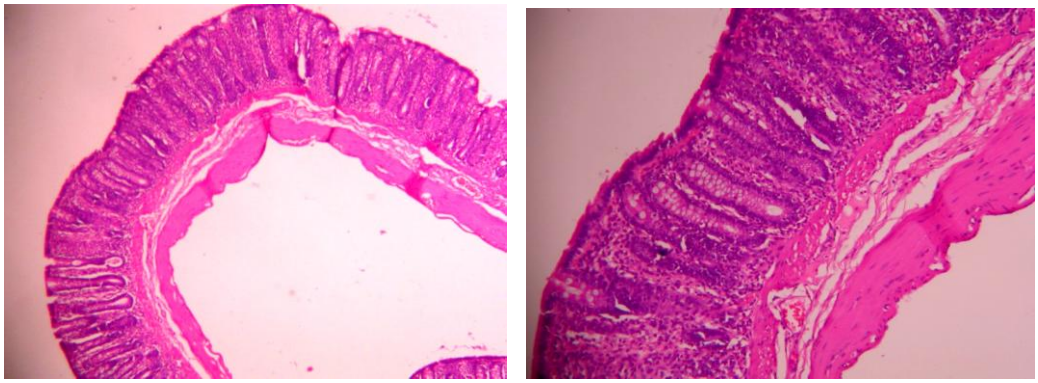
(A) **(B)**

Figure 4 Histopathoological sections of colonic mucosa in ENS 200mg/kg administered rat colon showing healing intestinal wall with inflammation limited to mucosa and submucosa. (Magnification; (a). 20X, (b). 100X)



(A) (B)

Figure 5 Histopathoological sections of colonic mucosa in ENS 400mg/kg administered rat colon showing healing intestinal wall with inflammation limited to mucosa.(Magnification; (a). 20X, (b). 100X)



(A) (B)

Figure 6 Histological appearance of SSZ 360mg/kg administered rat colon showing healing intestinal wall with reduced inflammation limited to mucosa.(Magnification; (a). 20X, (b). 100X)

DISCUSSION

Intracolonic administration of the TNBS in 35% ethanol results in long-lasting chronic ulceration and inflammation of the rat colon which is an established colitis model. This model of colitis was designed based on the principle that the ethanol vehicle would damage the colonic epithelium, thereby permitting entry of the hapten (TNBS) into the lamina propria where it would bind to tissue and act as an antigen [16]. Several features of this model make it attractive for the study of inflammatory bowel disease. Inflammation produce by TNBS in 35% ethanol is longer lasting with significant thickening of the

colonic wall is associated with cellular infiltration and ulcer and persisting for a long period of time [13].

In present study there was extensive colonic mucosal and submucosal damage characterized by infiltration of inflammatory cell and ulcer formation after administration of TNBS. Increase in colon weight /length ratio and MI of colonic tissue in TNBS administered ENS 100mg/kg and 200mg/kg, p.o treated group was reduced compared to group administered TNBS alone. However the histopathology also reveals the healing process in ENS treated colonic mucosa of rat which was similar to standard SSZ.

Myeloperoxidase is an enzyme found in neutrophils used as marker for inflammatory damage [17]. Myeloperoxidase is secreted by the neutrophils whenever there is inflammation and therefore the number of neutrophils is directly co-related with myeloperoxidase activity. Neutrophils play an important role in producing superoxide anion and a cascade of various reactive species leading to a very reactive hydroxyl and peroxide radicals [18]. Reduction in the activity of myeloperoxidase enzyme can be interpreted as a manifestation of the anti-inflammatory activity of a drug [17]. In present study there was an increase in MPO after administration of TNBS. ENS 200mg/kg and 400mg/kg, p.o showed significant reduction in the activity of MPO. The reduction in the MPO activity was confirmed histologically, since the level of leukocyte infiltration in the colonic mucosa was lower in ENS 200mg/kg and 400mg/kg, p.o treated groups compared to TNBS administered group. Hence this might be due to anti-inflammatory activity of ENS [12].

Oxidative stress and its consequent lipid peroxidation could aggravate free radicals chain reactions disrupting the integrity of intestinal mucosa barrier, and activate inflammatory mediators. It has been shown that colonic MDA contents increased and colonic SOD Levels decreased both in human and experimental animal studies [19, 20]. The levels of MDA were often used as an indication of oxidative damage and as a marker for free radicals-induced lipid peroxidation. In our study, ENS treated group showed significant reduction in malondialdehyde levels compared to TNBS-induced colitis group which is due to the inhibition of lipid peroxidation.

NO is associated with the initiation and maintenance of inflammation in IBD and that the selective inhibition of inducible NO synthase (iNOS) reduces the tissue damage [20,21]. Studies indicated that a high nitrate level appears to be secondary to the magnitude of inflammation [22]. In present study there was a significant increase in serum NO levels in TNBS induced colitis group compared to normal group, however there was significant decrease in ENS and SSZ treated groups.

Previous studies have suggested that excessive NO level could dilate vasculature, enhance vasopermeability, as well as inactivate the activity of antioxidases such as SOD, CAT, and GSH by means of reacting with hydrosulfide group (-SH) in the enzymes[23]. In present study, ENS treated group showed significant increase in anti-oxidant enzymes SOD, CAT and GSH compared to TNBS induced colitis group, suggesting its anti-oxidant activity. Results showed ENS acts by anti-inflammatory, anti-oxidant activity and lowering lipid peroxidation. Hence ENS is beneficial in experimentally induced IBD in rats.

CONCLUSION

Present study reveals that ENS possesses dose dependent anti-inflammatory and anti-oxidant properties comparable to standard sulfasalazine effects. This effect is due to its anti-inflammatory, anti-oxidant activity and inhibition of lipid peroxidation. Thus ENS has the protective effect in TNBS induced colitis in rats.

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