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HEALING EFFECT OF *TERMINALIA CHEBULA* FRUIT EXTRACT ON TRINITROBENZENE SULFONIC ACID INDUCED COLITIS IN RAT

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Abstract : The present study aims to evaluate healing effect of 50% ethanolic extract of *Terminalia chebula* fruit pulp (TCE) on trinitrobenzene sulfonic acid (TNBS, intra-colonic route) induced colitis in rats. TCE (600 mg/kg, oral) was studied in TNBS-induced colitis for its effects on fecal output, food and water intake and body weight changes, histology, antibacterial activity and levels of free radicals (nitric oxide and lipid peroxidation), antioxidants (superoxide dismutase; catalase and reduced glutathione) and acute inflammatory marker (myeloperoxidase) in colonic tissue. TNBS administration increased colonic mucosal damage and inflammation (macroscopic and microscopic) and stool output but decreased body weight which was reversed by TCE treatment. TCE showed significant antibacterial activity and enhanced the antioxidants but decreased free radicals and myeloperoxidase activities affected in TNBS colitis. Thus, Terminalia chebula dried fruit pulp extract healed colitis by promoting antioxidant status and decreasing intestinal bacterial load, free radicals and myeloperoxidase responsible for tissue damage and delayed healing.

Key words : *terminalia chebula* colitis free radicals antioxidants myeloperoxidase antimicrobial activity

INTRODUCTION

Ulcerative colitis (UC) is a major form of inflammatory bowel disease (IBD) which affects the colon and rectum and typically involves only the innermost lining or mucosa, manifesting as continuous areas of inflammation and ulceration, with no segments of normal tissue (1). Although the etiology remains largely unknown, it has been suggested that a combination of genetic susceptibility factors and the activation of the mucosal immune system or non-specific inflammatory reactions in response to luminal bacterial antigens along with persistent pathologic cytokine production

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contributes to the initiation and chronification of UC (2). Chronic inflammation due to chemical irritation, infection, or immune imbalance increases production of reactive oxygen species (ROS) and impairs antioxidant defenses, resulting in oxidative-stress (3). Activated neutrophils and macrophages are responsible for ROS or reactive nitrogen species (NOS) generation and the levels of ROS/NOS can be correlated with the severity of inflammatory changes in the colonic mucosa (4). Recently, we reported the healing effect of 50% ethanolic extract of dried fruit pulp of *Terminalia chebula* against acetic acid-induced colitis in rats (5).

Terminalia chebula (TC) is distributed in India, Nepal, China, Sri Lanka, Malaysia and Vietnam. It is called Haritaki in Hindi. It has been advocated in indigenous systems of medicine to treat many diseases such as parasitic infections, digestive diseases, irregular fevers, urinary diseases, flatulence, constipation, ulcers, vomiting and colic pain. It is reported to have antimicrobial, anti-inflammatory, antioxidant, immunomodulatory and adaptogenic properties (5). Acetic acid induces colitis by its direct necrotizing effect associated with acute inflammation and activation of arachidonic acid pathway while, TNBS (a hapten) induces a delayed-type hypersensitivity which proceed to develop colitis by chronic immunological inflammation followed by liberation of inflammatory markers like cytokines and arachidonic acid metabolites leading to oxidative stress, tissue damage and delayed healing (6). The present work evaluates the healing effect of TCE on another inducible model of intra colonic trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats.

MATERIALS AND METHODS

Collection of plant material and extraction

Fruits of TC were collected in the months of October to February from Ayurvedic Garden, Banaras Hindu University (BHU), Varanasi. The fruit pulps were cut into small pieces and dried at room temperature and powdered. The plants and their parts were identified with the standard sample preserved in the department of Dravyaguna, Institute of Medical Sciences, BHU, Varanasi. Fifty percent (50%) ethanol extract of TC (TCE) was prepared by adding 500 ml each of ethanol and distilled water in 200 g of dried fine powder of TC. The mixture is shaken at intervals and the extract was filtered after two days. The procedure was repeated twice at an interval of two days. The ethanol containing extract so obtained each time was mixed and later dried at 40°C in incubator. The yield was 28.3% (w/w). TCE was stored at –20°C until further use.

Animals

Inbred Charles-Foster strain albino rats (180-210 g) and Swiss albino mice (25-30 g) of either sex were obtained from the Central animal house of Institute of Medical Sciences, Banaras Hindu University, Varanasi. They were kept in the departmental animal house at 26°±2°C, 44-56% relative humidity and 10:14 h light and dark cycle for 1 week before and during the experiments. Animals were provided with standard rodent pellet diet (Pashu Aahar, Ramnagar, Varanasi) and water was given *ad libitum*. 'Principles of laboratory animal care' (NIH publication no. 82-23, revised 1985) guidelines were followed.

Approval from the Institutional Animal Ethical Committee was taken prior to the experimental work.

Chemicals

Sulfasalazine (Sazo, Wallace, Mumbai, India; SS) and 2,4,6-trinitrobenzene sulfonic acid (Sigma-Aldrich St. Louis, MO; USA) and other chemicals used in the study were purchased from standard companies. Muller-Hinton agar and broth (Hi-media, Mumbai, India), was used for antibacterial activity.

Induction of colitis

Colitis was induced by intra-colonic administration of TNBS to 24 hr fasted rat (6). Rats were either given intracolonic normal saline (NS, 0.4 mL/rat, negative control) or TNBS alone (40 mg/0.4 mL of 40% ethanol/rat, control).

Treatment protocol

TCE and standard UC protective drug, sulfasalazine (SS) were suspended in 0.5% carboxy methyl cellulose (CMC) in distilled water and were given orally, once daily in the volume of 1 ml/100 g body weight. Each group in the study contains 6 rats. First dose of CMC/TCE/SS was given 4 h after the induction of UC with TNBS and then the treatment was further continued for a period of 14 days. The rats were put on fast on 14^{th} day, after the oral administration of last dose of CMC (control), TCE (test extract) or SS (positive control) in TNBS induced colitis groups in rats while, rats with oral CMC plus intracolonic NS acted as negative control. TCE (600 mg/kg) and standard UC protective drug, SS (100 mg/kg) doses against TNBS-induced colonic mucosal damage score, weight and adhesions were selected on the basis of our earlier reported study on acetic acid induced colitis (5).

Assessment of diarrhoea

Diarrhoea was observed in intracolonic NS-treated (without colitis) and TNBS-induced colitis and after administration of oral CMC/TCE/SS. The effects were seen on the 7^{th} and 14^{th} day of the experiment. The result of TNBS was compared with NS while that of TCE/SS treated groups were compared with TNBS group.

Assessment of changes in body weight, food intake and water intake.

The above parameters were measured on the 7^{th} and 14^{th} day of the experiment. Each rat was individually weighed using standard rat weighing machine. Similarly a measured weight of enough food and water was given to each rat housed individually in the iron cages (8×11×7 cubic inches) at a fixed time of day and next day the amount of food and water left was calculated for individual rat.

Assessment of colonic damage and inflammation

All scorings of damage and excision of tissue samples were performed by an observer unaware of the treatment group. The rats in the various treatment groups were randomized before being sacrificed. The rats were weighed and sacrificed by an over dose of ether and proximal 8 cm of colon was removed. The colon was opened by a longitudinal incision, rinsed with tap water and pinned out on a wax block. Macroscopically visible damage was scored

on a 0-10 scale using the scoring system as described by Morris and associates (7), which takes into consideration these verity and number of ulcers in terms of tissue damage score, thickening and adhesions (signs of inflammation). Subsequently 8 cm of colon were taken for measurement of weight. The weight was expressed as mg/cm length of individual rat.

Histopathology of Colon

Histopathology of the colon was done in all the groups on 15th day of experiment to know the status of healing. Approximately 0.5 cm × 0.5 cm of colon was taken and fixed in 10% buffered formalin and paraffin embedded. 4-6 µm thick sections were stained with hemotoxylin and eosin stain for histological evaluation and examined under microscope at × 100 magnification.

Biochemical analysis

On 15th day of experiment the animals were sacrificed and colon of each rat was taken out and washed with cold normal saline. Antioxidants i.e. superoxide dismutase, SOD (8), catalase, CAT (9) and reduced glutathione, GSH (10); free radicals i.e. lipid peroxidase, LPO (11) and nitric oxide, NO (12); acute inflammatory marker, myeloperoxidase, MPO (13) and protein (14) were estimated in colonic mucosal homogenates following the standard procedures. LPO levels were estimated in terms of malondialdehyde (MDA) released during lipid peroxidation. Nitrites and nitrates are formed as end products of reactive nitrogen products during NO formation which are measured by using Griess reagent. SOD activity was estimated by its ability to inhibit reduction of nitro blue tetrazolium to blue coloured formazan in presence of phenazinemethasulphate (PMS) and NADH. One unit (U) of enzyme activity is defined as enzyme concentration required to inhibit the chromogen conversion by 50%. CAT measurement was done based on the ability of catalase to oxidize hydrogen peroxide. One unit (U) of catalase is the enzyme, which decomposes one mM of hydrogen peroxide per min at 25^{o}C. GSH activity in the homogenate was estimated by the ability of GSH to reduce DTNB within 5 min of its addition against a reagent blank with no homogenate. MPO activity was determined as an indicator of polymorphous nuclear leucocyte accumulation. MPO activity was estimated by its ability to inhibit reduction of nitro blue tetrazolium to blue coloured formazan in presence of phenazinemethasulphate (PMS) and NADH. One unit (U) of enzyme activity is defined as enzyme concentration required inhibiting the chromogen conversion by 50%.

Antimicrobial activity

In vitro antibacterial susceptibility test of TCE was done using serial concentrations of 50, 100, 150 and 200 mg/ml following the approved standards of the National Committee for Clinical Laboratory Standards (15) against various intestinal pathogens i.e. *Escherichia coli* ATCC 25922, *Shigellaboydii*, *Shigellasonnei* and *Shigellaflexneri* obtained from the American Type Culture Collection (ATCC) and clinical strain preserved at Department of Microbiology, Institute of Medical Sciences, BHU, Varanasi, India following the disk diffusion method while, minimum inhibitory concentration (MIC) was performed by micro dilution method (16).

Acute toxicity study in mice

Six adult Swiss albino mice of either sex (3 males and 3 females), weighing between 25 to 30 g fasted overnight, were used for acute toxicity study as per OECD guideline. Suspension of TCE was orally administered at 3 g/kg stat dose (5 times of the optimal effective dose of 600 mg/kg) to mice. Subsequent to TCE administration, animals were observed closely for first four hours, for any toxicity manifestation, like increased motor activity, salivation, convulsion, coma and death. Subsequently observations were made at regular intervals for 24 h. The animals were under further investigation up to a period of two weeks (17).

Statistical analysis

The statistical analysis was carried out by using unpaired t-test and one way analysis of variance followed by Dunnett's test for multiple comparisons. The values are represented as mean±SEM. $P<0.05$ was considered significant.

RESULTS

Effects on diarrhea

TNBS rats showed increase in faecal output from 2.33±0.13 g/100 g body weight at day 0 to 3.48±0.27 g/100 g body weight (49.4% increase) and 3.67±0.31 g/100 g body weight (57.5% increase, $P<0.01$) at day 7 and 14 respectively. TCE treated rats showed increase in faecal output from 2.46±0.21 g/100 g body weight at day 0 to 3.06±0.25 g/100 g body weight (24.3% increase) and 3.08±0.23 g/100 g body weight (25.1% increase) thus leading to decrease in fecal output from 49.4% to 24.3% (25.1% decrease) and 57.5% to 25.1% (32.4% decrease) from TNBS group at day 7 and 14 respectively. SS treated rats showed increase in faecal output from 2.16±0.19 g/100 g body weight at day 0 to 2.97±0.32 g/100 g body weight (37.4% increase) and 2.50±0.12 g/100 g body weight (15.8% increase), a decrease by 12.0% and 41.7% from TNBS group at day 7 and 14 respectively (Figure 1a).

Effects on body weight, food and water intake

TNBS-treated rats showed decrease in body weight from 199.4±2.19 g at day 0 to 182.8±3.77 g (8.3% decrease) ($P<0.01$) and 171.1±2.71 g (14.2% decrease) ($P<0.001$) at day 7 and 14 respectively. TCE treated rats on the other hand, showed increase in body weight from 191.3±5.13 g at day 0 to 213.9±4.19 g (11.8% increase) and 220.4±6.79 g (15.2% increase) ($P<0.01$) while, SS treated rats showed increase in body weight from 187.4±2.79 g at day 0 to 207.3±2.31 g (10.6% increase) and 218.1±3.76 g (16.4% increase) ($P<0.001$) at day 7 and 14 respectively (Figure 1b). However, little change was observed on food and water intake between the TNBS-treated and TCE and SS treated animals from 0 day to 14th day of study treatments.

Effects on colonic damage, inflammation and adhesions

Normal saline did not show any colonic mucosal damage, thickening or adhesion and the colonic weight was 158.3±6.37 mg/cm. TNBS increased colonic mucosal damage score (5.17±0.31, $P<0.001$), adhesions (5/6 rats, 83.3%) and weight to 248.8±6.67 mg/cm (57.2% increase, $P<0.001$) compared with normal saline group. TCE (600 mg/kg)

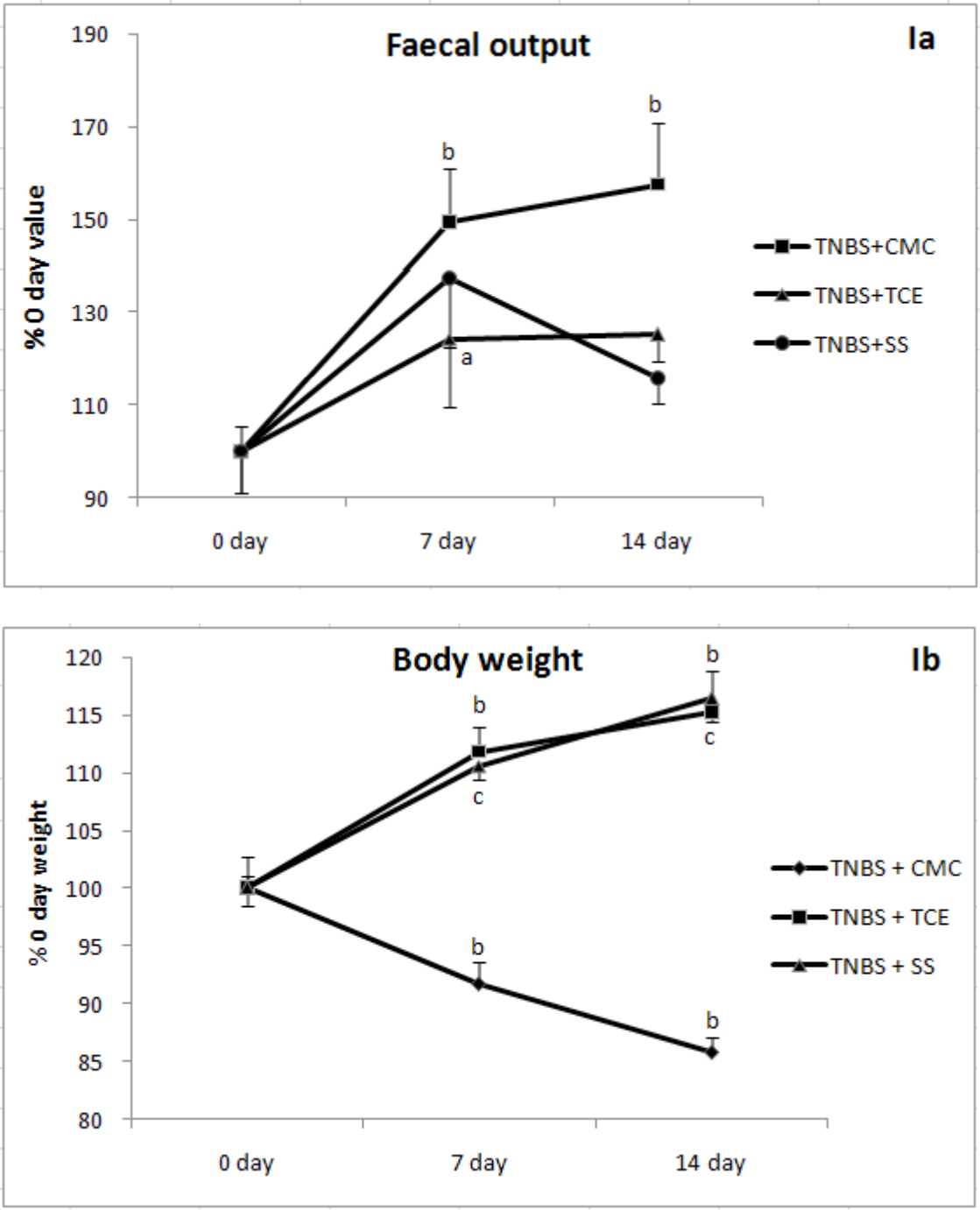

Fig. 1: Effects of TCE on TNBS-induced rat fecal output and body weight. Results are percent of mean±SEM of respective 0 day group value (n=6). [a]P<0.05, [b]P<0.01and [c]P<0.001 compared to respective 0 day group value % TNBS control.

reduced TNBS-induced colonic mucosal damage score by 61.3% (P<0.001), colonic weight by 27.0% (P<0.001) and adhesion by 60%. SS treated rats showed decrease in colonic damage score, colonic weight and adhesions by 77.4% (P<0.001), 33.5% (P<0.001) and 80.0% (1/6 rat) respectively compared with TNBS group (Figure 2).

Histopathology

Histology of colon of NS rats showed normal and clear structure with intact mucosa, submucosa and muscularis externa. TNBS colitis rats showed eroded mucosa, crypt destruction with severe cryptitis, lympho-plasmacytic infiltrate and transmural inflammation while, TNBS-induced colitis rats treated with TCE or SS showed improvement in the structures with near intact lamina propria with mild lympho-plasmacytic infiltrate and submucosa with mild lymphomononuclear aggregate (Figure 3a-d).

Effects on free radicals

TNBS enhanced both LPO and NO expressed as nmol/mg protein compared to NS rats. TCE and SS showed reversal of levels of both LPO and NO near to the NS level (Table I).

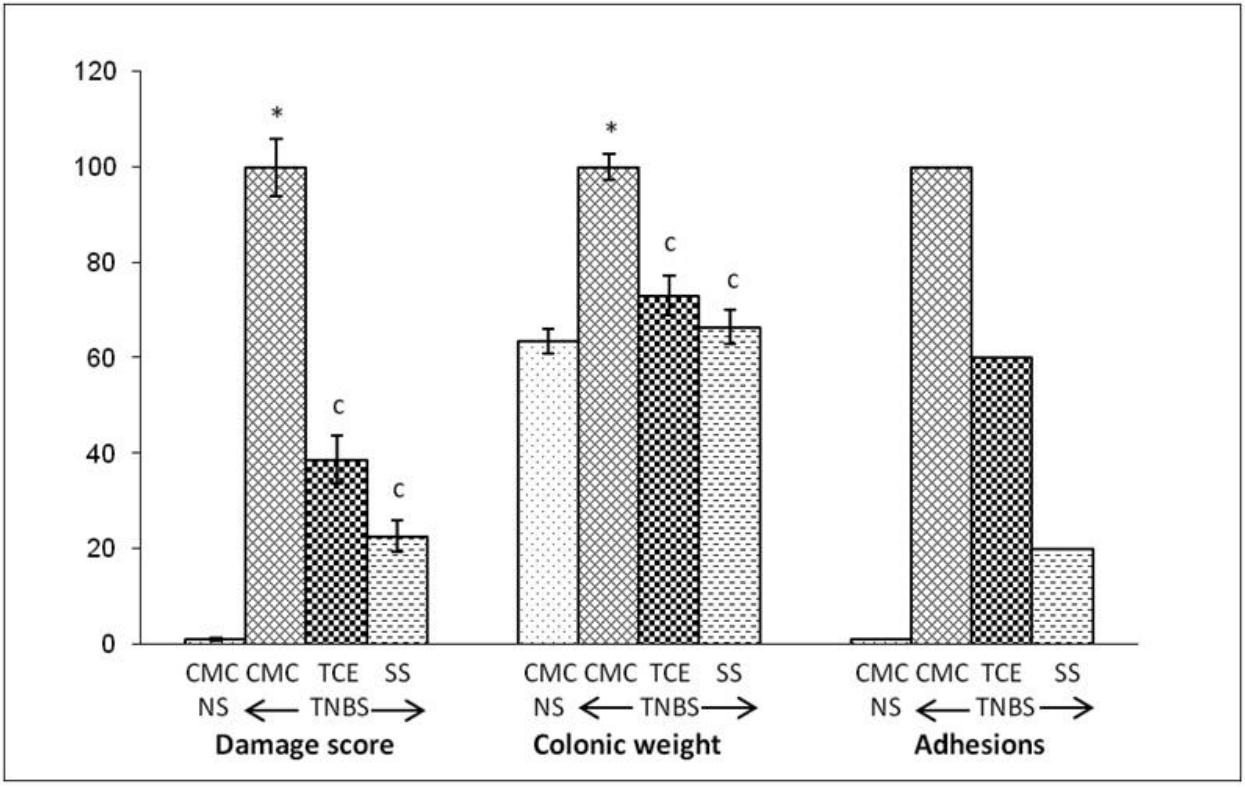

Fig. 2 : Effects of TCE on TNBS-induced rat colonic mucosal damage score, weightand adhesions. Results are percent of mean±SEM of respective TNBS control values (n=6). *P<0.001 compared to NS group (unpaired 't' test),and cP<0.001compared to respective TNBS group (one way analysis of variance followed by Dunnett's test).

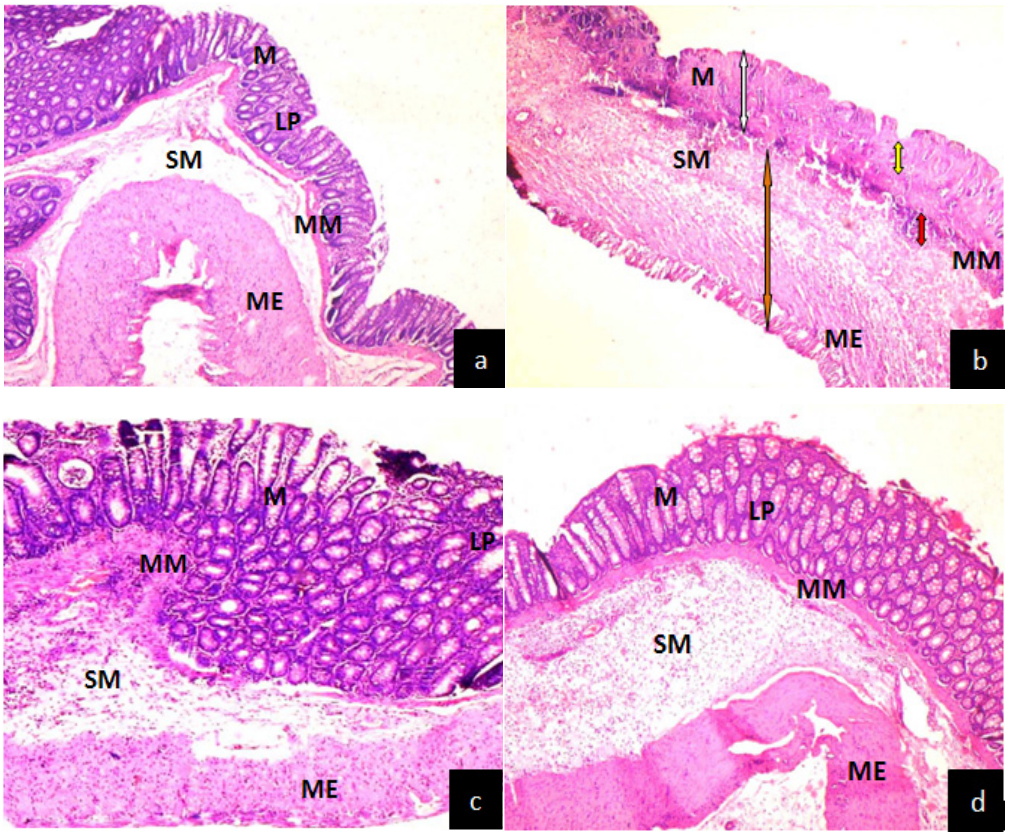

Fig. 3 : Histolgical section of rat colon stained with H & E stain (x100). **(a)** NS+CMC showing normal structure and clear with intact mucosa and sub mucosa. **(b)** TNBS+CMC showing ulcerated and eroded mucosa shown by white arrow, crypt destruction with severe cryptitis shown by yellow arrow, lymphoplasmacytic infiltrate shown by red arrow and transmural inflammation (predominantly-lymphocytes and plasma cells) shown by brown arrow. **(c)** TNBS+ TCE showing regenerative mucosa with mild crypt distortion and mild lympho-plasmacytic infiltrate in the lamina propria with oedematoussubmucosa and **(d)** TNBS+ SS showing intact mucosa with minimal lymphoplasmacytic infiltrate in the laminapropria. [M: Mucosa; SM: Submucosa; LP: Lamina propria; MM: Muscularis mucosa; ME: Muscularis externa].

Effect on antioxidants

TNBS treated animals showed significant decrease in SOD, CAT and GSH levels in the colonic mucosal incubates when expressed as mU (SOD and CAT) or nmol (GSH) per mg protein compared to NS group. TCE and SS treatments reversed the above changes in SOD, CAT and GSH levels in TNBS-induced colitis near to normal NS group (Table I).

Effect on myeloperoxidase

TNBS treated animals showed significant increase in MPO level in the colonic mucosal incubates when expressed as mU/ mg protein compared to normal NS rats. TCE and SS reversed the above changes in MPO level near to normal NS group (Table I).

Antimicrobial susceptibility and MIC

In-vitro antimicrobial susceptibility test

TABLE I : Effect of TCE and SS on TNBS-induced changes in free radicals (lipid peroxidation, LPO and nitric oxide, NO), antioxidants (superoxide dismutase, SOD; catalase, CAT and glutathione, GSH) and myeloperoxidase (MPO) in rat colonic mucosa.

Oral treatment (mg/kg, od × 14 days)	*Free radicals*		*Anti-oxidants*		*Myeloperoxidase*	
	LPO (nmol/mg protein)	*NO (nmol/mg protein)*	*SOD (mU/mg protein)*	*CAT (mU/mg protein)*	*GSH (nmol/mg protein)*	*MPO mU/mg protein*
NS+CMC	3.81±0.22	4.77±0.25	191.2±20.1	3.38±0.21	6.47±0.48	6.83±0.37
TNBS+CMC	10.50±0.62*	10.10±1.25*	443.7±10.5*	1.28±0.06*	3.61±0.27*	60.88±1.42*
TNBS+TCE 600	7.00±0.24[b]	7.09±0.49[c]	390.3±30.2[c]	3.30±0.18[c]	7.58±0.47[c]	20.03±1.23[c]
TNBS+SS 100	4.78±0.64[c]	5.57±0.39[c]	232.4±10.6[c]	3.19±0.22[c]	5.35±0.21[c]	16.96±2.19[c]

Results are mean±SEM (n=6). *P<0.001 compared to respective NS+CMC group (unpaired 't' test) and [b]P<0.01, [c]P<0.001 compared to respective TNBS group (one way ANOVA followed by Dunnett's test).

TABLE II : *In-vitro* antibacterial activity and minimum inhibitory concentration (MIC) of TCE.

Name of organism	*TCE Antibacterial activity (Zone of inhibition in mm)*				*MIC (mg/ml)*
	(50 mg/ml)	*(100 mg/ml)*	*(150 mg/ml)*	*(200 mg/ml)*	
E. coli ATCC 25922	7.1±0.57	8.3±0.34	9.0±0.43	10.1±0.92	6.25
Shigellasonnie	8.3±0.94	9.2±0.81	9.8±0.45	11.3±0.81	1.57
S. boydii	8.0±0.47	9.4±0.63	10.1±0.41	11.5±0.65	1.57
S. flexneri	8.7±1.41	9.8±0.35	10.6±0.62	12.1±0.87	0.79

Values are mean±SEM of 3 experiments in each group.

against gram negative intestinal bacteria like *E. coli*, *S. boydii*, *S. sonnie* and *S. flexneri* with TCE (200 mg/ml) as indicated by zone of inhibition >10 mm. However, the MIC value against *E. coli, S. sonnie, S. boydii* and *S. flexneri was* 6.25, 1.57, 1.57 and 0.79 mg/ml respectively indicating more susceptibility of *S. sonnie* and *S. boydii* to lower concentration of TCE (Table II).

Acute toxicity study

TCE did not show any acute toxicity manifestation like increased motor activity, salivation, colonic convulsion, coma and death in mice, observed up to a period of two week.

DISCUSSION

TNBS-induced colitis led to loss of body weight and increase in diarrhea/faecal output, which could be due to alterations in epithelial function produced, either directly or indirectly by products released from activated mast cells (18). Our present study, further indicated significant increase in colonic mucosal damage score, adhesions and colonic weight and crypt destruction with cryptitis, eroded mucosa, lymphoplasmacytic infiltrate and transmural inflammation, due t o TNBS-induced immunological inflammatory changes. TCE-treated rats showed reversal of above effects thus, indicated healing effect in TNBS-induced colitis. These effects may

be attributed to the anti-inflammatory, immunomodulatory and antiulcer properties of TC as reported earlier (19-21).

Neutrophils infiltration is one of the most prominent histological features in the inflamed colonic mucosa of colitis and source of myeloperoxidase (22). We found a several fold increase of myeloperoxidase in TNBS-induced colitis and reduction of MPO activity can be interpreted as a manifestation of the anti-inflammatory effect of TCE. Activated neutrophils produce ROS/NOS within intestinal mucosa inducing oxidative stress, which plays a significant role in the pathogenesis of UC/IBD (23-26). These chemicals attack the cell membranes and produce damaging fatty acid radicals and lipid hydroperoxides. Therefore, elimination of ROS could be an important strategy in healing of UC and antioxidants hasten it by destroying free radicals (27). TCE possessed significant antioxidant activity which helped in preventing oxidative damage and promoted healing. The above effects of TCE could be attributed to various active constituents namely phenolic compounds, triterpinoids, tannins, and flavonoids which are commonly known for their antioxidant activity (28-31).

Extensive immune activation and breakdown of the intestinal barrier provides bacteria access to the gut mucosal immune system, resulting in uncontrolled inflammation and dysbiosis (32). TCE exhibited considerable level of inhibition against the intestinal organisms as reported earlier for herbal products (33) and this could be due presence of certain phytochemical including flavonoids in TCE. The antibacterial effect could be contributory factors in helping healing of colitis induced by TNBS. TCE was also found to have no acute toxicity even with five times of the optimal effective dose administered to mice indicating its safety on use.

The results of the present study with 50% ethanol extract of fruit pulp of *Terminalia chebula* do indicate promising healing effects in TNBS-induced colitis. It further, substantiates our earlier reported work on its healing effect against acetic acid induced colitis in rats.

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