Curcumin half analog modulates interleukin-6 and tumor necrosis factor-alpha in inflammatory bowel disease

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ABSTRACT

Background: The present study was aimed at examining the effect of dehydrozingerone (DHZ), half analogue of curcumin which is the active constituent of turmeric (*Curcuma longa*) in the di-nitrochlorobenzene (DNCB) induced model for inflammatory bowel disease (IBD). **Materials and Methods:** Male Wistar rats (200–220 g) were divided into four groups ($n = 6$). Chemical induction of IBD was done by sensitizing with 300 µL of 20 g/L of DNCB (in acetone) onto the nape of rats for 14 days followed by intra-colonic instillation of 250 µL of DNCB (0.1% DNCB in 50% alcohol) solution on day 15. Rats in Group 1 (normal control) and Group 2 (DNCB control) were treated with vehicle. Rats in Group 3 were treated with DHZ (100 mg/kg, *p.o.*; 8 days) and Group 4 animals were treated with sulfasalazine (SS) (100 mg/kg, *p.o.*; 8 days). On 24th day, the rats were killed, colon removed and the macroscopic, biochemical, and histopathological evaluations were performed. **Results:** The levels of myeloperoxidase, thiobarbituric acid reactive substrate, and nitrite increased significantly ($P < 0.05$) in the DNCB group whereas reduced significantly in the DHZ and SS treated groups. Serum nitrite levels were found to be insignificant between the different groups. Interleukin-6, tumor necrosis factor-alpha level was significantly high in the DNCB group. **Conclusion:** These findings show that DHZ can be a promising molecule for the treatment of IBD.

Key words: Dehydrozingerone, di-nitro chlorobenzene, interleukin-6, myeloperoxidase, thiobarbituric acid reactive substrate, tumor necrosis factor-alpha

INTRODUCTION

Inflammatory bowel disease (IBD) with its two presentable forms Crohn's disease (CD) and ulcerative colitis (UC) is a chronic inflammatory disorder of gastrointestinal tract. The key etiological factors include environment,[1-6] familial,[7] immunological,[8] and genetic in origin.[9,10] Moreover, the mucosal tissue damage in UC/CD patients which is mediated by free radical and reactive oxygen species (ROS) also plays a key role in the progression of the disease.[11,12] Apart from the above, recently some drugs are also known to cause inflammatory bowel like diseases.[13] The present approved drug therapy which is limited to immunity and microbes provoked IBD have a lot of toxicity limited usage.[14,15] Hence,

for those who are unresponsive to standard therapy, the use of medicinal plants or their active ingredients, particularly nontoxic food additives have been increased in the recent times. But the lack of sufficient scientific understanding related to their mechanism of action limits their mainstream medical usage.[16] Turmeric, an Indian spice which is widely used as a flavoring and coloring agent and is derived from the rhizomes of the medicinal plant *Curcuma longa*. The active moieties include curcumin (diferuolyl methane), demethoxy curcumin, and bisdemethoxy curcumin.[17] Among these, curcumin displays wide range of pharmacological activities such as anti-oxidant,[18] anti-microbial,[19] anti-inflammatory,[20] and free radical scavenging properties[21] along with the one responsible for its yellow color imparting property. The anti-inflammatory property of curcumin in IBD has been proved in Trinitrobenzenesulfonic acid (TNBS) induced mouse colitis[16] and di-nitrochlorobenzene (DNCB) induced colitis,[22] whereas dehydrozingerone (DHZ) which is also a potent, synthetic, half-analog of curcumin[23] with proved anti-oxidant,[24] free radical scavenging,[25] and

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anti-inflammatory properties[26] has not yet been studied in IBD. Hence, the present study involves the anti-inflammatory activity along with mucosal healing of DHZ in DNCB induced IBD.

Of several models, the use of DNCB as a haptenizing agent has been well-characterized as it resembles human IBD by eliciting T-cell mediated immunological response when bound to the proteins of colon and induce colitis.[27] In the present study, we estimated the levels of myeloperoxidase (MPO), thiobarbituric acid reactive substrate (TBARS), tissue nitrite, interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) and histological findings in the colon.

MATERIALS AND METHODS

Materials
DNCB, 2-thiobarbituric acid, trichloroacetic acid was obtained from (HiMedia Laboratories Pvt. Ltd., Mumbai, MH, India). DHZ (prepared in-house), O-dianisidine dihydrochloride, Griess reagent was obtained from Sigma–Aldrich, St. Louis, MO, USA. IL-6 and TNF-α kit was obtained from Life Technologies, Carlsbad, CA, USA. Total protein kit was obtained from Thermo Fisher Scientific Inc., Waltham, MA, USA. Analytical grade chemicals were used.

Preparation of dehydrozingerone
DHZ was prepared using vanillin, acetone, and sodium hydroxide in a similar manner[28] in which 2.5 g of vanillin was dissolved in 20 ml of acetone. To the resulting solution, 50 ml of 1N solution of NaOH was added drop-wise from a burette with continuous stirring for about half an hour and the stirring was continued for about 2 h. Excess of acetone was evaporated at room temperature by keeping it undisturbed overnight. The solution was acidified with 2N HCl at ice cold conditions until a yellow precipitate was formed. Then it was filtered and dried. Recrystallization was done with absolute alcohol. The formed product was confirmed as DHZ by checking the melting point and gas chromatography mass spectrography.

Animals
Healthy inbred male albino rats of Wistar strain (220–250 g) were used. The rats were kept in an air-conditioned room maintained at a temperature of 23°C ± 2°C with a 24 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/Kasturba Medical College/88/2011–2012) and were carried according to the guidelines of the committee for the purpose of control and supervision of experiments on animals, Government

of India. Intra-rectal administration of DNCB was carried out under diethyl ether anesthesia.

Induction of colitis and treatments
The protocol and dosing strategy was taken from the previous study.[22] 24 animals were divided into four groups of six animals each as follows-normal control, DNCB control, DHZ treated, and sulfasalazine treated (SS). On day 1, hair on the nape of the rats was removed by applying 10% sodium sulfide (Na₂S). After 2 h, 300 µL of DNCB in acetone (20 g/L) was applied on the same spot with a microtiter pipette. From day 2–14, spraying of DNCB onto the nape of rats was continued. Day 14, animals were fasted overnight. On day 15, 250 µL of DNCB (0.1% DNCB in 50% alcohol) solution was instilled into the rectum of the rat using an intravenous cannula of 16 G around 6–8 cm from the anus. From day 16–23, control received 0.3% carboxymethyl cellulose (*p.o*), DHZ group received DHZ (100 mg/kg, *p.o*) and SS group received SS (100 mg/kg, *p.o*). Finally, on day 24, the animals were killed by cervical dislocation and dissected open to remove the colon. The entire colon starting from caecum was taken and placed on a slab for measuring the length and weight. Around 6–7 cm of the proximal part of the 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 the proximal colon was taken for histopathological study and stored in 10% formalin until the histological studies were carried out. Before killing the animals, blood was collected, 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 11.5 g/L solution of potassium chloride 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 myeloperoxidase
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.[29] The entire estimation was carried out in a 96-well plate, and the readings were taken on an microplate reader (ELx 800, BioTek Instruments Inc., Winooski, VT, USA) at 490 nm. 50 µL of sample was taken in duplicate. To this, 250 µL of o-dianisidine hydrochloride (ODA)-H₂O₂ 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% of H_2O_2 in 1 ml of ODA solution. After addition, the reading was noted at 5 min, 15 min. After this, 4M 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 the standard plot which uses horseradish peroxidase as standard. Note: H_2O_2 and ODA solutions are light sensitive, so wrapped in aluminum foil. The entire experiment was done under dark conditions especially the addition of ODA-H_2O_2 solution.

Assay of lipid peroxides in colonic homogenates

Malonaldialdehyde (MDA) which was formed by the breakdown of polyunsaturated fatty acids serves as an index for determining the extent of peroxidation reaction.[30] To 250 μL of colonic homogenate, 250 μL of 2-thio barbituric acid-trichloroacetic acid (TBA-TCA) reagent was added. The reagent comprises 15% (w/v) of TCA; 0.375% (w/v) of TBA; 15 mg of butylated hydroxy toluene; 200 μL of 0.25 N hydrochloric acid. The solution was kept in a sonicator for half an hour 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 about 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, tetramethoxy propane.

Nitrite assay

During inflammation, macrophages and neutrophilic granulocytes of intestinal mucous membrane are activated and release large amounts of toxic NO,[31] which would damage the intestinal mucous membrane or even react with superoxide anion (O_2^-) and produce more active oxidizing substance called oxidized nitrous acid ($OONO^-$).[32] 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 mucous membranes as a barrier is destroyed. In this assay, 100 μL of sample (serum or colonic tissue homogenate) was taken and to this 100 μL of Griess reagent was added in a 96-well plate. The absorbance was measured at 540 nm by placing the plate undisturbed in the dark for 10 min. The concentrations were calculated with the standard plot using sulfanilamide as standard.

Tissue interleukin-6/tumor necrosis factor-alpha estimation

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

Histopathological studies

All the rats of the normal control group, DNCB group, DHZ treated, and SS treated groups were sacrificed on the day of 24 from the initial day of sensitization by light ether anesthesia followed by carotid bleeding. A small portion of the proximal colon was selected for histopathology. The specimens were stored in 10% neutralized buffered formalin, and processed for histopathological findings.

Statistics

The results were expressed as mean ± standard error of mean. Statistical significance was calculated by analysis of variance followed by *post-hoc* Tukey's multiple comparison test by using Prism 5.03 (GraphPad Software Inc., La Jolla, CA, USA). $P < 0.05$ was considered to be significant.

RESULTS

Body weight

Figure 1 shows a significant decrease in the mean of end weight as percentage of initial weight in DNCB, DHZ groups when compared to control at $P < 0.05$ which were found to be 93.83 ± 1.29%, 93.55 ± 3.35%, respectively. The SS treatment group did not show any significant reduction in the body weight.

Colon weight

From Figure 2, it can be seen that there is a significant increase in the weight of the colon in the 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 DHZ treated group at $P < 0.05$ which was found to be 1.30 ± 0.03 g.

MPO estimation

Figure 3 depicts a significant rise in the 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 (DHZ and SS) compared to DNCB group at $P < 0.05$ which were found to be 3.08 ± 0.13, 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

It is evident from Figure 4 that when compared to the control group, there was a significant rise in the levels of tissue nitrite at $P < 0.05$ in the DNCB group which was found to be 1.40 ± 0.07 ng/μg of protein. Compared to DNCB, both DHZ and SS treated groups showed a significant decrease in the levels of tissue nitrite which were found to be 0.91 ± 0.05 and 0.85 ± 0.03, respectively.
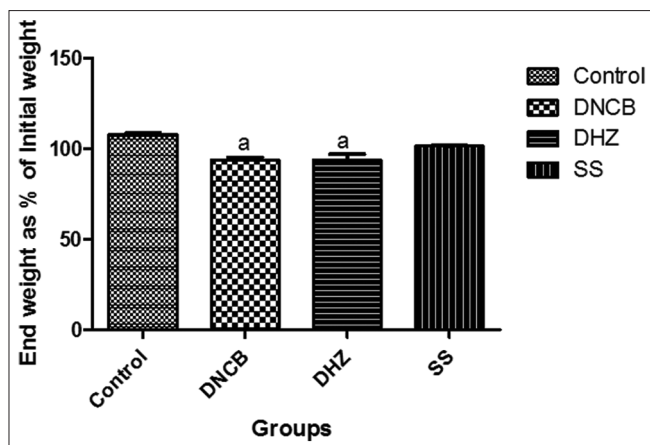
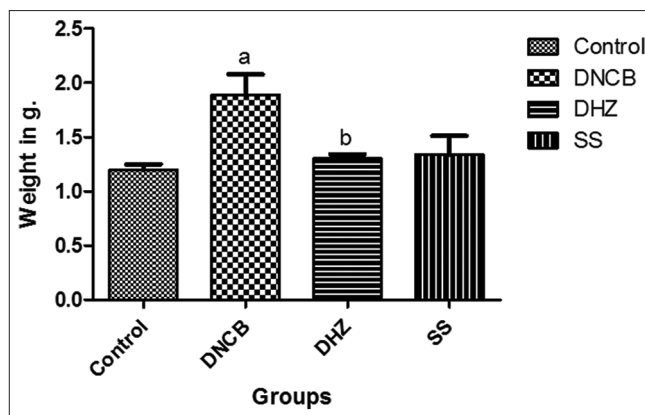**Figure 1:** Effect of dehydrozingerone and sulfasalazine on body weight a: *P* < 0.05 when compared with control

Figure 2: Effect of dehydrozingerone and sulfasalazine on weight of colon. a: *P* < 0.05 when compared with control; b: *P* < 0.05 when compared with di-nitrochloro benzene
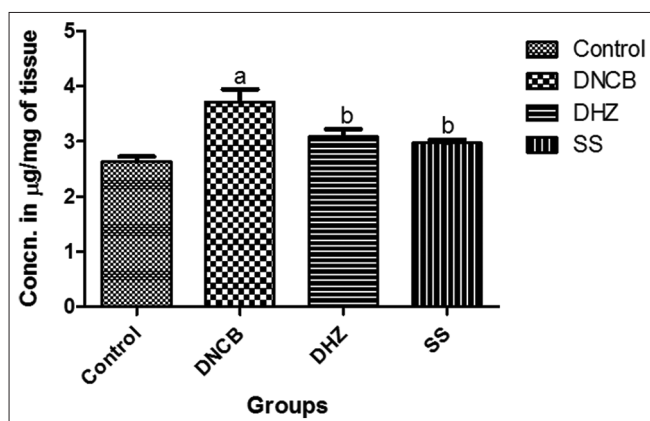

Figure 3: Effect of dehydrozingerone and sulfasalazine on the levels of myeloperoxidase in tissue homogenates. a: *P* < 0.05 when compared with control; b: *P* < 0.05 when compared with di-nitrochloro benzene
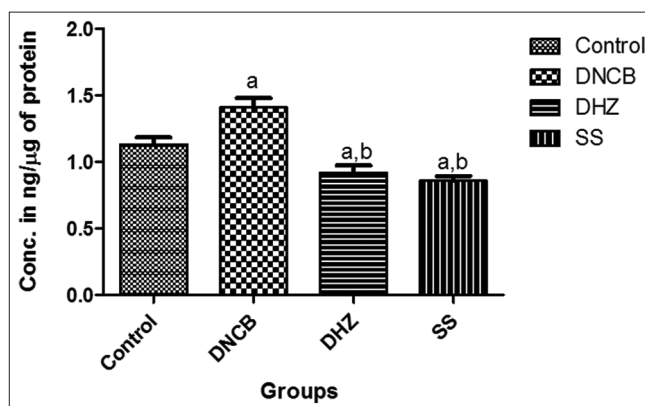

Figure 4: Effect of dehydrozingerone and sulfasalazine on tissue nitrite concentrations. a: *P* < 0.05 when compared with control; b: *P* < 0.05 when compared with di-nitrochloro benzene

Thiobarbituric acid reactive substrate estimation

Figure 5a exhibits a significant rise in the levels of MDA in the DNCB group at *P* < 0.05 which was 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 DHZ and SS treatment group which were found to be 0.355 ± 0.040, 0.327 ± 0.043 nm/mg of protein, respectively, at *P* < 0.05.

Estimation of interleukin-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 (DNCB only), 0.065 ± 0.0076 (DHZ), 0.0286 ± 0.004 (SS), respectively. When compared with DNCB group, none of the groups showed a significant decrease in the levels except the standard SS treatment group as shown in Figure 5b.

Estimation of tumor necrosis factor-alpha in the colonic tissue homogenates

From Figure 5c, it can be seen that there was a significant rise in the levels of TNF-α in the DHZ 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 were found to be 0.015 ± 0.00193 (DNCB only), 0.021 ± 0.00022 (DHZ treated), 0.014 ± 0.00079 (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 were expressed as the concentration of nitrite in μg and also percentage decrease with respect to control in the serum. As shown in Figure 5d, when compared to control, none of the groups showed a significant decrease in the levels of nitrite in the serum.
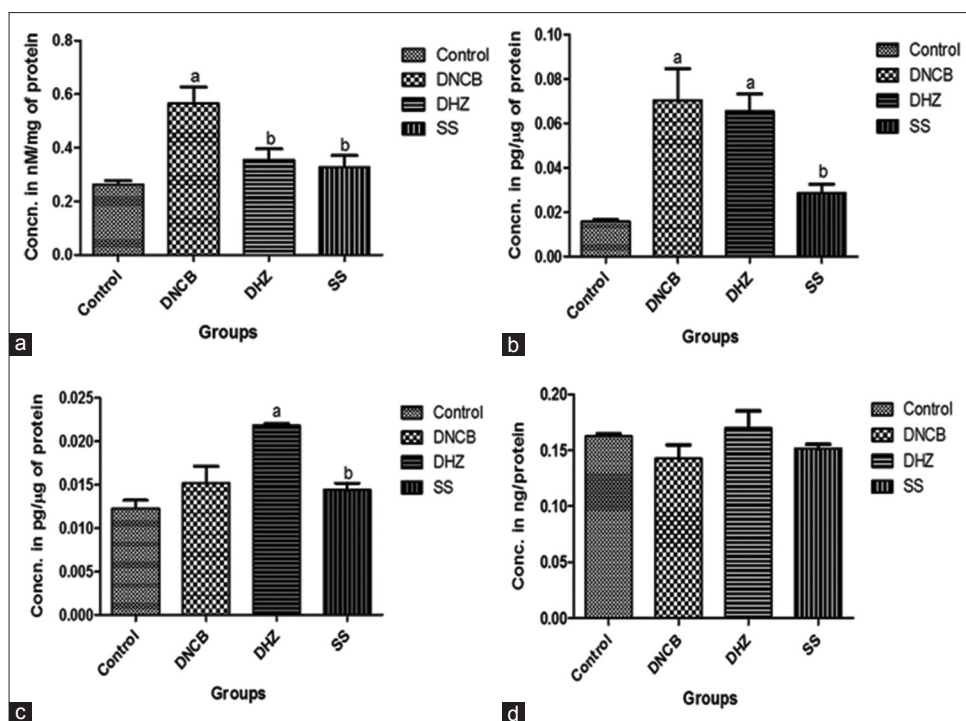
Figure 5: (a) Effect of dehydrozingerone and sulfasalazine on the concentration of malonaldialdehyde in the colonic tissue homogenates. a: $P < 0.05$ when compared with control; b: $P < 0.05$ when compared with di-nitrochloro benzene. (b) Effect of dehydrozingerone and sulfasalazine on the levels of IL-6. a: $P < 0.05$ when compared with control; b: $P < 0.05$ when compared with di-nitrochloro benzene. (c) Effect of dehydrozingerone and sulfasalazine on the levels of tumor necrosis factor -alpha. a: $P < 0.05$ when compared with control; b: $P < 0.05$ when compared with di-nitrochloro benzene. (d) Effect of dehydrozingerone and sulfasalazine on serum nitrite concentration

Histopathological studies

The normal histology of the colon was noted in the control: (a) In the DNCB treated group, colonic ulceration and congestion were clearly seen. Crypt abscesses were observed in the muscularis propria (b) DHZ and SS treated groups were similar to control (c and d, respectively) depicting normal crypts and a few inflammatory cell infiltration [Figure 6].

DISCUSSION

IBD is a result of complex interactions between a quartet of host-derived and external elements that involve various aspects of the intestinal microbiota, the immune system, the genetic composition of the host, and specific environmental factors.[33] In IBD, the ultimate goal is to provide relief to the patients by modifying the above-mentioned factors. Apart from these factors, ROS also play an important role in the progression of the disease.[34,35] Hence, DHZ was chosen, as it has proved anti-inflammatory,[26] anti-oxidant,[24] and wound healing[23] activities, but its role in chemically induced models of IBD has not been found out.

DNCB by acting as a chemical hapten is capable of inducing delayed-type hypersensitivity when applied
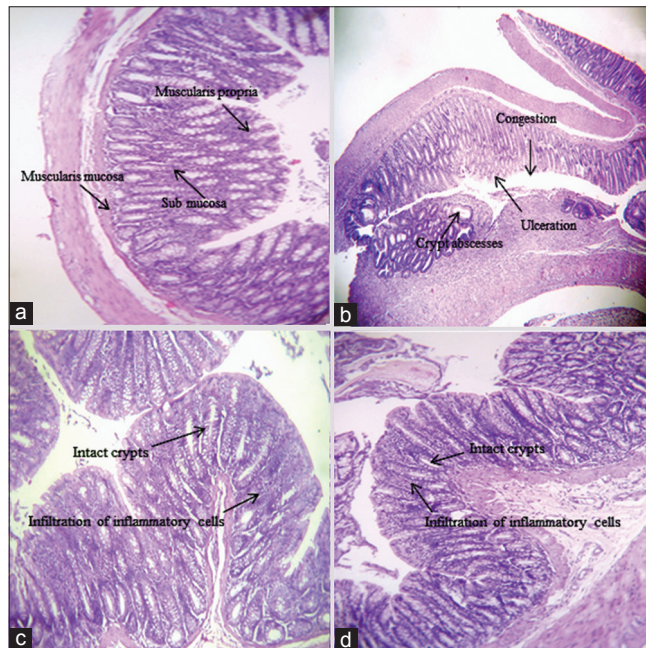

Figure 6: Histopathological studies, A: Normal control; B: Di-nitrochloro benzene; C: Dehydrozingerone; D: Sulfasalzaine

to the skin or intestinal. In this model of colitis, animals were presensitized to develop colitis, where the sensitivity is passively transferred by lymphocytes[36] and immune-suppression will mitigate the bowel injury.[37,38]

This model also shares some of the histological features associated with idiopathic human colitis.[39]

In the present study, the antioxidant effect of DHZ was assessed on the basis of gross parameters (body weight, colon weight, and colon length) and biochemical parameters (MPO, lipid peroxidation, nitrite, IL-6, and TNF-α).

There was a significant reduction in the body weight of DNCB and DHZ treatment group when compared to control. Intracolonic instillation of DNCB causes abdominal pain and anorexia which in turn causes the weight loss.[40]

The weight of colon is raised due to inflammation and also because of the increased activity of the fibroblasts leading to the overgrowth of muscularis mucosa.[41] DNCB group showed a significant rise in the weight of colon which was significantly ameliorated in the DHZ treatment group.

In the animal models of intestinal inflammation, to evaluate the drug activity and also to measure the extent of disease severity, myeloperoxidase activity is generally evaluated.[29] In our experiment, myeloperoxidase activity in the inflamed colon was determined. The drug DHZ was able to produce a reduction in the MPO activity, which can be considered as an anti-inflammatory property of the test compound in the DNCB model.

In the present study, the lipid peroxides were found to be significantly raised in the DNCB induced group which is an indicative of oxidative stress. The test drug was able to encounter the oxidative stress by reducing the colonic tissue contents of lipid peroxides. The undetectable levels of GSH were supported by earlier reports.[42]

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,[43] rather than being systemic. The decreased levels of nitrite in the treatment group indicated the mucosal protective effect of DHZ on the colon which was further confirmed with histopathology findings.

There was a significant increase in the tissue IL-6 levels after intra-colonical challenge. The raised cytokine levels were unable to be ameliorated by DHZ, which indicated that the drug might act through ROS pathway. Because of its effective anti-oxidant[24] and free radical scavenging properties,[25] it was able to suppress MPO,[44] TBARS, NO rather than cytokine suppression. 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 installation. The drug DHZ 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 nonimmune cells.[43]

The histopathological findings suggest that DHZ is able to protect the colonic mucosa from ROS like nitrite, peroxide, etc., and these findings are in line with the previously reported protective effects of sesamol on DNCB-induced colitis.[45]

CONCLUSION

We may conclude that the drug dehydrozingerone showed an activity comparable to that of sulfasalazine and protected the mucosa from the deleterious effects of DNCB.

ACKNOWLEDGMENTS

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