

## Effect of Manuka honey and sulfasalazine in combination to promote antioxidant defense system in experimentally induced ulcerative colitis model in rats

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Manuka honey (MH, 5g/kg) provided protection against trinitro-benzo-sulphonic acid induced colonic damage. Combination therapy (MH+sulfasalazine) also reduced colonic inflammation and all the biochemical parameters were significant compared to control and MH alone treated group. Combination therapy showed additive effect of the MH which restored lipid peroxidation and improvement of antioxidant parameters. Morphological and histological scores were significantly reduced in combination groups. In inflammatory model of colitis, oral administration of MH (5g/kg) and combination with sulfasalazine (360 mg/kg) with MH (5g/kg) significantly reduced the colonic inflammation. The results indicate the additive effect of Manuka honey with sulfasalazine in colitis.

**Keywords:** Antioxidant defense system, Monuka honey, Sulfasalazine, Ulcerative colitis

Ulcerative colitis (UC) is characterized by the presence of acute or chronic inflammation of digestive system mostly colon. Patients with ulcerative colitis are at high risk of developing colorectal carcinoma, however, the etiology of UC is still unknown. In this condition inflammatory involvement, distal to the splenic flexure was more prominent. This is because of increased free radical production, resulting from respiratory burst of infiltrating phagocytic cells and impaired antioxidant defense<sup>1</sup>. Responsive inflammatory mediators include reactive oxygen species (ROS) and cytokines, which contribute to the inflammatory cascade in modulating the immune system of UC. Pathological changes for chronic intestinal inflammation, found as a result of innate immune signals, such as Th1 cytokine involving release of IL-1, IL-6, TNF- $\alpha$  and the activation of Toll-like receptors (TLRs)<sup>2-4</sup>.

Among several inflammatory mediators, TNF- $\alpha$ , which is induced, synthesized and secreted from macrophages, lymphocytes, and polymorphonuclear neutrophils, is regarded as the most prominent "first-line" cytokines<sup>5,6</sup>. Moreover, TNF- $\alpha$  has overlapping and synergetic activities to induce the production of transcription factors like nuclear factor- $\kappa$ B (NF- $\kappa$ B)

and other cytokines. TNF- $\alpha$  stimulates and induces the production of other inflammatory mediators such as ROS. It also activates oxidative stress-responsive genes which amplify and prolong inflammation during UC including the loss of balance between the oxidant and antioxidant which was thought to be a fine balance under normal physiological conditions. Excessive production of ROS in mucosal cells induced by inflammatory and immune responses could directly or indirectly cause damage of intestinal epithelial cells, subsequently influences the mucosal integrity or initiate an inflammatory signaling cascade and lead to severe impairment in experimental colitis<sup>7,8</sup>.

The aim of treatment for UC is the induction and maintenance of remission of symptoms and mucosal inflammation. Hence, treatment of UC is difficult because of its complex etiology. Although, drugs like, 5-aminosalicylic acid (5-ASA), sulfasalazine (SASP), and glucocorticoids could inhibit the inflammatory mediators through different mechanisms locally or systemic, these agents are limited in their use due to involvement in the down-regulation of the immune and inflammatory responses of UC including adverse reactions during prolonged treatment and high relapse rate. It is important that effective drugs with fewer adverse reactions should be developed to prevent UC from initiating and relapsing.

Honey is a powerful antioxidant and used in the day to day life as natural sweetener<sup>9</sup>. Importantly, it contains varieties of enzymes such as oxidase, invertase, amylase, Catalase etc. Manuka honey (MH) is obtained from the plant *Leptospermum scoparium*, (family Myrtaceae) and used therapeutically for its antibacterial properties. Recent studies suggest that MH has ameliorative properties in wound healing, fungal infections, ophthalmic disorders, diabetes, gastrointestinal tract disorders, skin ulcers and infections.<sup>10,11</sup>

Present study has been undertaken to evaluate the combined effect of Manuka honey with sulfasalazine in TNBS-induced colitis model and its effect on myeloperoxidase, lipid peroxidation, glutathione level and antioxidant enzymes levels in rats.

### Materials and Methods

**Materials**—Manuka honey (MH) was obtained from Comvita New Zealand Ltd, New Zealand. Hexadecyl-trimethylammonium bromide buffer (HTAB), *O*-dianisidine dihydrochloride, glutathione reductase, nicotinamide adenine dinucleotide phosphate reduced (NADPH) and thiobarbituric acid (TBA) were purchased from Sigma Chemical (St. Louis, MO, USA). Phosphate buffered saline (PBS), Tris-HCl buffer and ethylenediamine tetrachloroacetic acid (EDTA), were purchased from M/s HiMedia Chemicals (Mumbai, India). Reduced glutathione, hydroxylamine hydrochloride, trichloroacetic acid, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and nitroblue tetrazolium (NBT) were procured from M/s Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). The pellet diet duly approved by the Institute's Animal Ethics Committee was obtained from M/s Ashirwad Industries (Punjab, India).

**Animals and treatment**—Pathogen-free young male Wistar rats weighing 150–250g were obtained from the Central Animal House of the Institute. Animals were divided into following 5 groups of 6 animals each. Group I (ethanol as vehicle), Group II (TNBS-20mg in 35% ethanol, administered through rectum as single intra-colonic application into descending colon through a rubber catheter, Group III (MH at a dose of 5 g/kg body weight), Groups IV (sulfasalazine 360 mg/kg body weight), as a positive control) and Group V (MH 5 g/kg body weight + sulfasalazine 360 mg/kg body weight). Animals were allowed free access to water and a normal pellet diet. They were housed in polypropylene cages bedded with sterilized rice husks under 12:12 hr light and dark cycles. The

experimental design was approved by the Institute's Animal Ethics Committee (IAEC).

**Induction of ulcerative colitis**—The ulcerative colitis was induced in male Wistar rats weighing 150–250g as per Morris *et al*<sup>12</sup>. Briefly, animals were anaesthetized with diethyl ether followed by a single intra-colonic application of 20 mg TNBS dissolved in 0.25ml of 35% ethanol in saline (v/v), referred as TNBS-ethanol into the descending colon. Control rats received same volume of 35% ethanol diluted with saline (v/v). Animals were sacrificed under ether anesthesia 14 days after TNBS-ethanol administration and colonic mucosa was obtained for evaluation of mucosal changes (microscopically) and antioxidant status.

**Tissue preparation**—The tissue (colonic mucosa) were excised and perfused with ice-cold perfusion solution (0.15M KCl, 2 mM EDTA, pH 7.4). Tissues were homogenized in Tris-HCl buffer (50 mM, pH 7.4), and the homogenates were centrifuged at 10,000 g at 4°C for 30 min to obtain post-mitochondrial supernatant (PMS). The PMS was used for the estimation of myeloperoxidase (MPO)<sup>13</sup>, malondialdehyde (MDA)<sup>14</sup>, reduced glutathione-(GSH)<sup>15</sup>, superoxide dismutase (SOD)<sup>16</sup>, catalase (CAT)<sup>17</sup> and glutathione peroxidase (GPx)<sup>18</sup>.

**Assessment of inflammation**—The distal 10 cm of the colon were excised soon after rat sacrifice. Adherent adipose tissue was made free and colon incubated into Tris-buffer for 30 min at 37°C in shaking water bath (1ml/100g tissue). Colon was dissected longitudinally into three pieces for morphologic, histological analysis and antioxidant assay.

**Morphological analysis**—Morphological assessment for the inflammation was done according to Enteritis gross morphological score<sup>19</sup>: 0=no inflammation sign in the whole of 10 cm of intestine; 1=slight inflammation, slight redness, villi visible under 15 fold magnification; 2=intermediate inflammations, discontinuous hyperemia intermediate redness of villi; and 3=intensive inflammation, hyperemia, intensive redness of villi.

**Histological analysis**—Microscopic examination was done by a qualified pathologist using hematoxylin and eosin staining in a blinded fashion as described by Levine *et al*<sup>20</sup>. After pharmacological intervention, all group's were histopathologically assessed by using following score; 0 = normal; 1 = mild mixed infiltrates in the lamina propria; 2 = focal

superficial ulceration of the mucosa only, moderate cryptitis and crypt abscess; 3 = deep ulceration penetrating colonic wall through mucosa till muscularis mucosa and severe inflammation; 4 = necrosis through large bowel wall.

**Statistical analysis**—The data were entered into the data base programme and the statistical significance of differences between various groups was determined by using two way analysis of variance (ANOVA) for multiple comparison using Students-Newmann-Keul's (SNK) procedure and Dunnett's procedure was used for data analysis with respect to control. *P* values <0.05 were considered statistically significant.

## Results

During the experimental period of 14 days there was no mean weight loss/gain and none of the rats were excluded from the study for any reason. Two rats died in the TNBS group at the early stage, the reason was unclear but rats were replaced with the other two rats so that there is no variation in the groups.

After induction of inflammation in the colon, diarrhea was observed in about half the animal population and the behaviour was aggressive with restlessness, whereas, the rats continued to have diarrhea for 6-7 days in all the groups. Most of the animals had local peritonitis with transmural necrosis and inflammatory masses in large colon.

**Morphological observations**—On day 15 after TNBS challenge, the morphology of colon revealed inflammatory change in the mucosa. The distal 10 cm was assessed for inflammation as gross inflammatory index according to scoring system. The morphological score in the TNBS groups was significantly increased ( $P < 0.001$ ) as compared to control group animals (Fig. 1). MH treatment significantly reduced ( $P < 0.05$ ) the morphological inflammatory changes as compared to TNBS group animals (Fig. 1) and also in sulfasalazine (SSZ) treatment group inflammation was observed significantly reduced compared to TNBS group (Fig. 1). Combination of sulfasalazine and MH effectively decreased the morphological changes as compared to sulfasalazine and MH alone treated groups.

**Histological findings**—Microscopic examination of the colon tissue showed presence of inflammation in the mucosa which was scored as per Levine A<sup>20</sup>. In the control group, there was no significant changes ( $P < 0.001$ ), but reactive lymphoid follicles were present (Fig. 2). The intact mucosa inflamed lamina

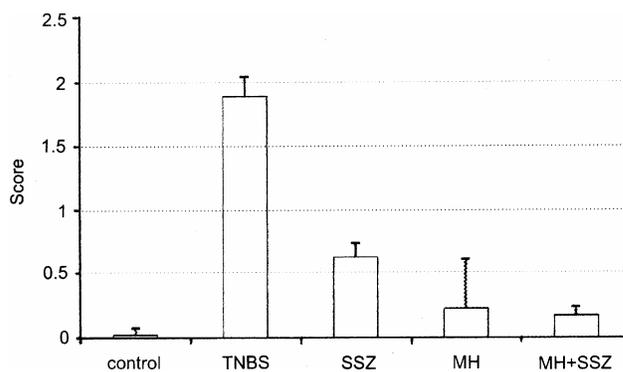


Fig. 1—Effect of Manuka honey (MH) and sulfasalazine (SSZ) on the morphological observations after 14 days of single dose treatment of TNBS-induced colitis in rats. [Values are mean±SD from 6 animal in each group, \* $P < 0.001$  considered significant with respect to control, #  $P < 0.05$  considered significant with respect to TNBS group.]

propria and widened sub-mucosa. Vehicle treated group showed mixed inflammation, neutrophils and eosinophils in the lamina propia (Fig. 3) and lymphocytic infiltration into the crypt with feature of cryptitis (Fig. 4a, b). Severe inflammation of colon was observed and histological score was also very high as compared to vehicle treated group. In MH group the tissue showed mild inflammation of lamina propia by lymphocytes and scattered eosinophils. There was no evidence of cryptitis and no crypt abscess was seen (Fig. 5). The sulfasalazine treated group showed mild cryptitis and the infiltration of the eosinophils in the lamina propia indicate mild to moderate persistent inflammation associated with leucostasis (Fig. 6). In the combination group (MH+sulfasalazine), the pathological changes showed the incremental observation, presence of crypt distortion indicating the persistent inflammation (Fig. 7). There was mild inflammation of lamina propia with occasional neutrophils. No cryptitis was found in combination group. The histological score was maximally reduced in combination group ( $P < 0.001$ ).

**Biochemical observation**—Significant alterations were observed in all antioxidant enzyme and non-enzyme levels after the Manuka honey, sulfasalazine and combination treated group (Table 1).

## Discussion

Previous experimental models of TNBS-induced colitis was used to study the acute and chronic mucosal damage of the colon, and the biochemical phenomena associated with free radical production<sup>21-23</sup>. TNBS metabolism *in vivo* is performed either by enzymatically or non-enzymatically by ascorbate to

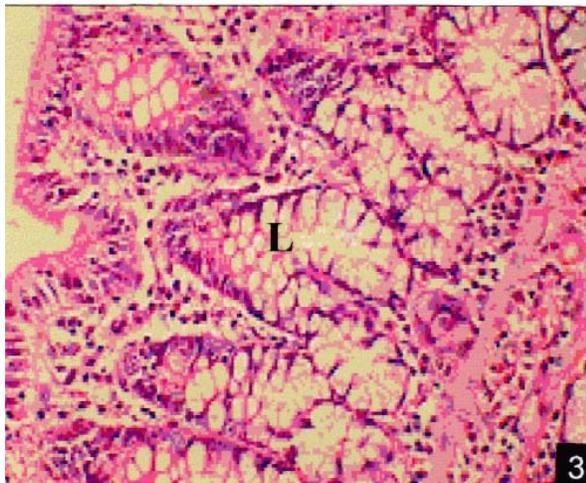
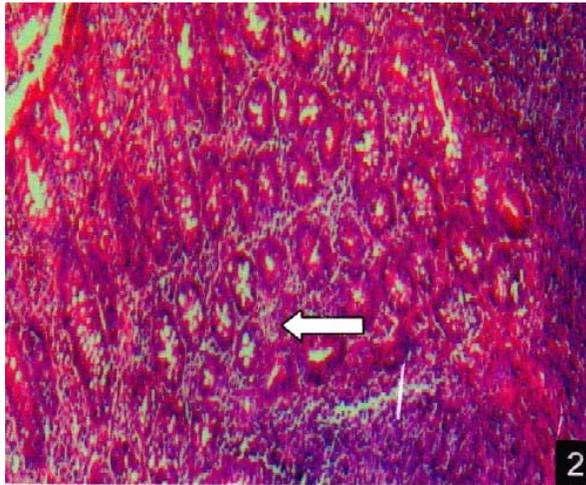


Fig. 2—The normal lymphoid follicles in the vessel of colon tissue. (H&E,  $\times 280$ ). Fig. 3—Moderate inflammation in the lamina propria of colon in the vehicle (ethanol) treated group. [L= lamina propria, the arrows indicate the infiltration of eosinophils, neutrophils (H&E,  $\times 280$ )

produce superoxide anion ( $O_2^-$ ), and hydrogen peroxide ( $H_2O_2$ )<sup>23</sup> suggesting that TNBS-induced colitis may be partially mediated by cytotoxic reactive oxygen metabolites<sup>24,25</sup>. The other sources of TNBS-induced ROS production by the inflamed colonic mucosa include the mitochondrial and microsomal transfer chains, xanthine oxidase, cyclo-oxygenase, and lipoxygenase enzymes<sup>26-28</sup>. ROS can also be produced by the phagocytic cells through NADPH pathway and by the overproduction of pro-inflammatory mediators (i.e., cytokines and arachidonate metabolites). It has been suggested that neutrophils are predominantly responsible for production of excessive amount of ROS by the tissue in colonic inflammation<sup>29,30</sup>. MPO is a good marker of inflammation following tissue injury. Significant increase in MPO activity was observed after TNBS treatment. The reduction in MPO activity after MH treatment indicate a reduction in the number of neutrophils within the mucosa, as evident from the histological studies, suggesting restoration of the normal homeostasis.

Cellular antioxidant enzymes such as SOD, CAT, and GPx and GSH (free radical scavengers) protect cells and tissues against noxious free radicals. Impaired antioxidant defense system after TNBS challenged colonic inflammation has been reported. Also it was found that CAT, SOD, and GPx present in very small amount<sup>21-33</sup>.

In the present study, the activity of SOD was decreased significantly which may have led to the inefficient removal of  $O_2^-$  radicals from the cellular milieu, resulting in the ROS burden. However these radicals overproduction has an inhibitory effect on the

Table 1—Effect of combination of Manuka honey and sulfasalazine on MDA, MPO, SOD, CAT, GSH, and GPx level following experimentally induced UC model, on day 15.

[Value are mean $\pm$ SD from 6 animals in each group]

|     | Group I          | Group II         | Group III         | Group IV           | Group V           |
|-----|------------------|------------------|-------------------|--------------------|-------------------|
| MDA | 0.94 $\pm$ 0.26  | 3.22 $\pm$ 0.48* | 1.03 $\pm$ 0.35*# | 1.06 $\pm$ 0.08*#  | 1.72 $\pm$ 0.34*# |
| MPO | 0.49 $\pm$ 0.12  | 2.98 $\pm$ 0.25* | 1.05 $\pm$ 0.27#  | 1.08 $\pm$ 0.13#   | 0.48 $\pm$ 0.31*# |
| SOD | 3.38 $\pm$ 0.61  | 1.70 $\pm$ 0.24* | 3.27 $\pm$ 0.82*# | 3.27 $\pm$ 0.83#   | 3.38 $\pm$ 0.60*# |
| CAT | 2.68 $\pm$ 0.30  | 0.92 $\pm$ 0.19* | 1.58 $\pm$ 0.30#  | 1.2367 $\pm$ 0.19# | 1.12 $\pm$ 0.23#  |
| GSH | 0.24 $\pm$ 0.0   | 0.09 $\pm$ 0.01* | 0.19 $\pm$ 0.02#  | 0.18 $\pm$ 0.01#   | 0.18 $\pm$ 0.01#  |
| G P | 12.54 $\pm$ 0.72 | 4.36 $\pm$ 0.59* | 11.09 $\pm$ 1.11# | 10.53 $\pm$ 1.19#  | 12.03 $\pm$ 2.14# |

P value: \* <0.001 with respect to control, # <0.05 considered with respect to TNBS group

Group I: Control; Group II: TNBS group; Group III: MH group; Group IV: Sulfasalazine group; Group V: MH + sulfasalazine group

MDA = malondialdehyde (nmoles of TBARS formed/min/mg protein); MPO = myeloperoxidase ( $\mu$ moles of  $H_2O_2$  consumed/min/mg protein); SOD = superoxide dismutase (IU/mg protein); CAT = catalase ( $\mu$ moles of  $H_2O_2$  consumed/min/mg protein); GSH = reduced glutathione ( $\mu$ g/mg protein); GPx = glutathione peroxidase (IU/mg protein)

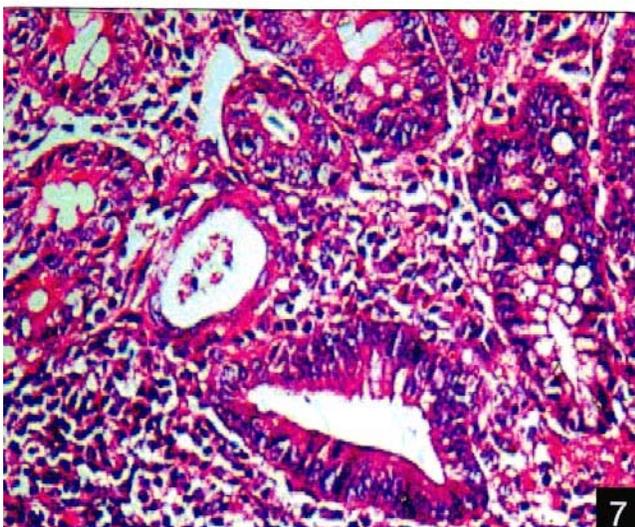
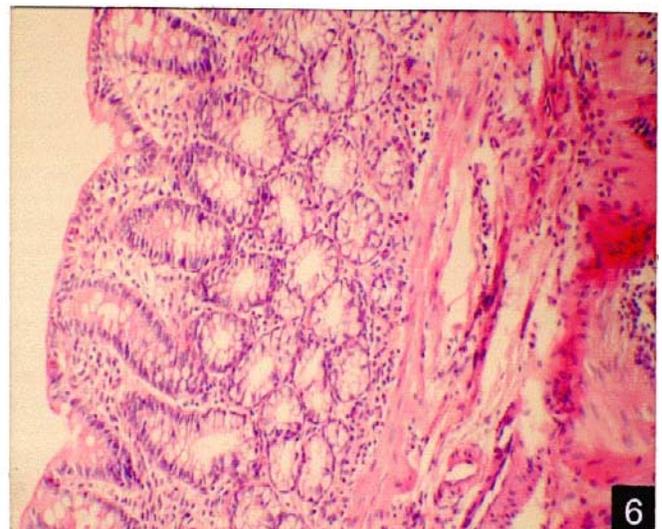
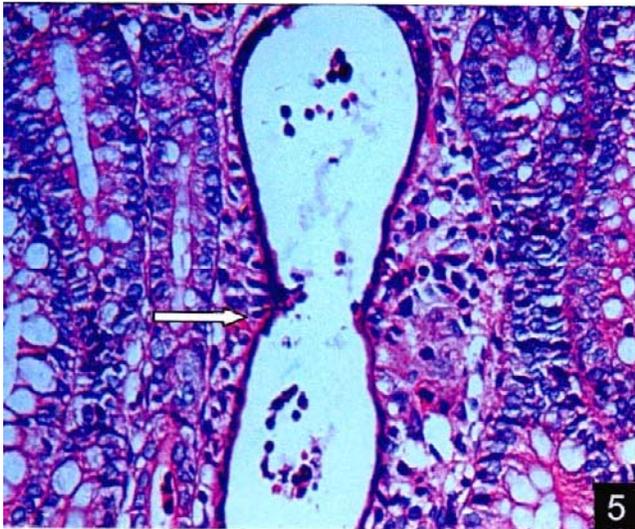
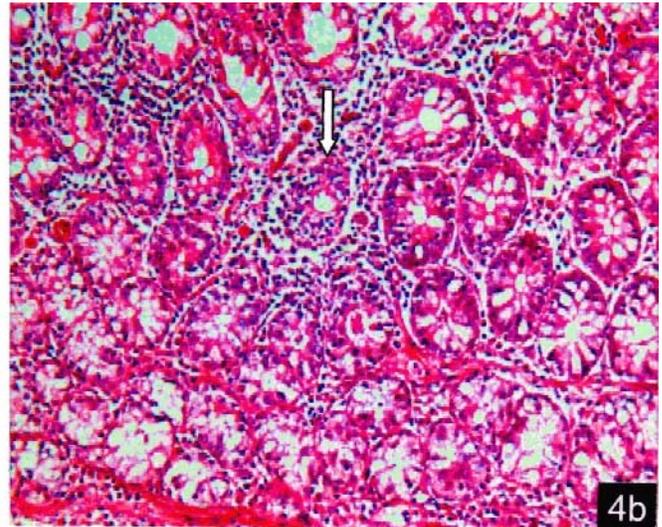
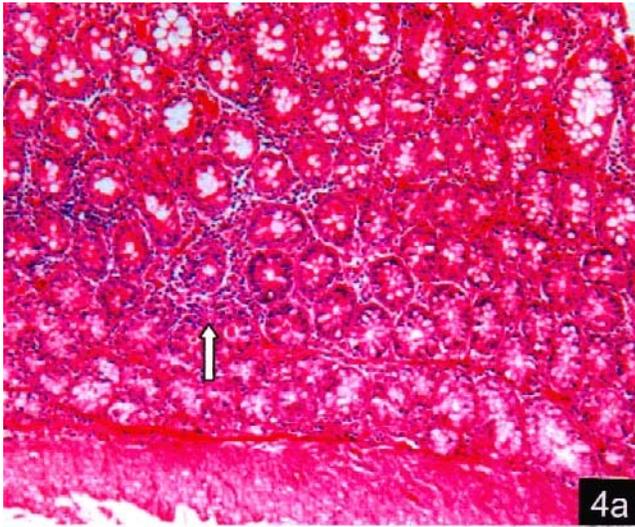


Fig. 4—(a) Mild chronic inflammation in lamina propria and focal cryptitis of the colon after 14 days of the single dose treatment of TNBS in rats. Arrows indicate the infiltration of inflammatory cells. (H&E  $\times 280$ ). (b) High power microphotograph showing cryptitis of the colon after 14 days of the single dose treatment of TNBS in rats. Arrows indicate the infiltration of inflammatory cells (H&E,  $\times 550$ ).

Fig. 5—Treatment of Manuka honey shows cystic dilatation of the crypt with abscess in the colon of rats after 14 days of the single dose treatment of TNBS (H&E  $\times 550$ ). Arrows indicate the infiltration of inflammatory cells (H&E,  $\times 550$ ).

Fig. 6—Treatment of sulfasalazine showing leucostasis in the vessel of the rats colon after 14 days of the single dose treatment of TNBS (H&E  $\times 280$ ).

Fig. 7—Combined treatment of Manuka honey and sulfasalazine showing crypt abscess and crypt distortion of the rats colon after 14 days of the single dose treatment of TNBS (H&E  $\times 280$ ).

antioxidant enzymes responsible for removal of ROS such as CAT.

In the inflammatory condition like TNBS induced colitis, the natural balance between ROS production and protective free radical scavengers may be disturbed, leading to tissue injury. Present results also showed regulation of free radical and other ROS after the TNBS treatment, whereas the antioxidants like SOD, CAT, GPx were down regulated. These parameters were ameliorated following MH combination treatment.

Disturbed tissue protection (free radical scavengers and ROS production) makes the cells more susceptible to oxidative damage that may contribute to the development of the lesions observed in this model. Thus, increased oxidative stress and impairment of antioxidant defenses may contribute to the pathogenesis of UC. The present results with TNBS-induced lipid peroxidation are consistent with those of Loquercio et al<sup>34</sup>. However, in addition, in the present study, significant decrease in oxidant enzyme activities and additionally increased lipid peroxidation were observed. Using the TNBS-induced colitis model, significant enhancement in the activities of antioxidants such as SOD, CAT, GPx and reduced glutathione was observed after treatment with Manuka honey and with combination sulfasalazine.

Following treatment with the Manuka honey and sulfasalazine SOD was significantly improved as compared to the control rats similarly study by Zhou et al,<sup>35</sup> reported that SOD level was elevated after the reduced inflammation. Han et al,<sup>36</sup> hypothesized that TNBS induced macroscopic and microscopic damages with increase in MPO activity, and intense immunostaining for nitrotyrosine were reduced by bovine SOD.

Catalase (CAT) is an enzyme found ubiquitously in nearly all living organisms and catalyses the decomposition of toxic hydrogen peroxide to water and oxygen. In the TNBS induced inflammatory UC model, it is found that CAT level decreased significantly than the control group. After treatment with Manuka honey and sulphasalazine its level found elevated but the combination group increased more significantly CAT level than alone treated groups. SOD activity was at peak and reached more than their control values after treatment with the Manuka honey as compared to CAT. Non-enzymatic antioxidants, reduced glutathione is the most abundant endogenous

thiol in eukaryotic cells which has been observed to be enhanced after Manuka honey treatment. Glutathione is found almost exclusively in its reduced form, since its oxidized form glutathione disulfide (GSSG), glutathione reductase, is constitutively active and inducible only upon oxidative stress. GSSG plays a pivotal role in maintenance of balance of cellular reduction and oxidation (redox) reactions and acts as radical scavenger due to redox-active sulphhydryl groups directly reacting with oxidants. Lowered glutathione content has generally been considered as an 'indicator' of increased formation of ROS, and glutathione depletion in mammalian cells causes 'cell damage by oxidative stress'<sup>37</sup>

A depletion of GSH can lead to increased lipid peroxidation or malondialdehyde which is the end-product of lipid peroxidation and in this process reactive oxygen species degrade polyunsaturated lipids<sup>38</sup>. In the present study, the glutathione level increased significantly after Manuka honey and sulfasalazine treatment suggesting its protective effect in the colon. GPx activity was very high and reached to their control values after treatment with the Manuka honey and sulfasalazine as compared to CAT or other antioxidant parameters, indicating that these are the primary enzymes which play a major role in the detoxification of ROS. In present study, combination of Manuka honey and sulfasalazine showed more beneficial effect than the individual treated groups.

In conclusion, present results indicate, both Manuka honey and sulfasalazine act by reducing the oxidant load at the inflammatory site and improved UC in both alone and combination treatment with MH and sulfasalazine, however combination treatment showed more beneficial.

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