

## Ginkgo biloba attenuates mucosal damage in a rat model of ulcerative colitis

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### Abstract

Intestinal inflammatory states, regardless of specific initiating events, share common immunologically mediated pathways of tissue injury and repair. The efficacy of various drugs used to treat ulcerative colitis (UC) was investigated. The aim of the present study is to evaluate the effects of ginkgo biloba extract on the extent and severity of UC caused by intracolonic administration of acetic acid in rats. The inflammatory response was assessed by histology and measurement of myeloperoxidase activity (MPO), reduced glutathione (GSH), tumor necrosis factor (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) levels in colon mucosa. Oral pretreatment with Ginkgo biloba in doses of (30, 60, 120 mg kg<sup>-1</sup> body weight) and sulfasalazine in a dose of (500 mg kg<sup>-1</sup> body weight used as reference) for 2 days before induction of colitis and continued for 5 consecutive days, significantly decreased colonic MPO activity, TNF- $\alpha$ , and IL-1 $\beta$  levels and increased GSH concentration. Moreover, Ginkgo biloba attenuated the macroscopic colonic damage and the histopathological changes-induced by acetic acid. These results suggest that Ginkgo biloba may be effective in the treatment of UC through its scavenging effect on oxygen-derived free radicals.

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### 1. Introduction

Indirect evidence suggests that free radicals and excited-state species play a key role in both normal biological function and in the pathogenesis of certain human diseases. Generation of activated species by inflammatory cells is a major microbicidal mechanism and may also mediate important components of the inflammatory response [1]. The etiology of ulcerative colitis (UC) is still unknown. However, genetic factors in combination with environmental factors are suggested to be involved in the pathogenesis [2]. Prolonged or inadequate activation of the intestinal immune system plays an important role in the pathophysiology of chronic mucosal inflammation [3]. Furthermore, infiltration of neutrophils, macrophages, lymphocytes and mast cells, ultimately giving rise to mucosal disruption and ulceration [4]. The infiltrated and activated neutrophils represent an important source of reactive oxygen and nitrogen species. These

species are cytotoxic agents, inducing cellular oxidative stress by cross-linking proteins, lipids, and nucleic acids, causing cellular dysfunction and damage [5]. In addition to free radicals, neutrophils can also release proteases and lipid mediators that can contribute to intestinal injury [6].

Macrophages produce certain cytokines, such as tumor necrosis factor (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ), the levels of which are often increased in both animal models and patients with ulcerative colitis [7,8]. In addition, IL-1 $\beta$  appears to be a primary stimulator of diarrhea, the major symptom of intestinal inflammation [9]. Interleukin-1 $\beta$  and TNF- $\alpha$ , are key immunoregulatory cytokines that amplify the inflammatory response by activating a cascade of immune cells. IL-1 $\beta$  and TNF- $\alpha$ , secreted by activated macrophages, stimulate production of cytokines, arachidonic acid metabolites, and proteases by intestinal macrophages, neutrophils, smooth muscle cells, fibroblast, and epithelial cells. An important effect of IL-1 $\beta$  not shared by TNF- $\alpha$ , is its ability to induce IL-2 and IL-2 receptor on T lymphocytes [10].

Ginkgo biloba is one of the oldest herbal medicines that have been used as a therapeutic agent in modern pharmacology. It

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is mainly recommended for the treatment of peripheral arterial disease and cerebral insufficiency in the elderly [11,12]. Several constituents of Ginkgo biloba are biologically active such as flavonoids, terpenoids and Fe-SOD that are likely to be responsible for the wide-ranging therapeutic benefit of the plant [13]. Recently, the antioxidant properties of Ginkgo biloba have been examined as a potential mechanism for its beneficial action [14]. Ginkgo biloba extracts show protective effects against free radical-mediated damage in biological systems, including ischemia-reperfusion injury of the brain, heart and retina [15–17] and in cyclosporine A-induced lipid peroxidation [18]. Furthermore, it had been reported that Ginkgo biloba had a beneficial effect in Alzheimer's disease and showed minimal side effects [19].

The role of Ginkgo biloba in the possible modulation of colon inflammation had not been verified. This prompted us to study the potential effects of Ginkgo biloba extract on experimental acetic acid-induced colitis in rats. Since, oxygen-free radicals, neutrophils and proinflammatory cytokines are clearly involved in the pathogenesis of inflammatory bowel disease, the inflammatory response was assessed histologically and biochemically. In addition, IL-1 $\beta$  and TNF- $\alpha$  were also measured.

## 2. Materials and methods

### 2.1. Chemicals

Ginkgo biloba was purchased from Pharma Nord ApS (Vejle, Denmark), sulphasalazine, 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), adenosine diphosphate, agents for myeloperoxidase assay were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A). All other chemicals used were of analytical grade.

### 2.2. Animals

Fifty-six adult male Wistar albino rats (150–200 g) were used throughout this work (supplied from the Animal Care Centre, King Saud University). The animals were maintained in a room under standard conditions of light, feeding and temperature. The study was conducted in accordance with the standards established by the guide for the care and use of laboratory animals of the College of Medicine Research Centre (CMRC) at King Saud University. The rats were housed individually in a rack mounted with wire mesh cages to prevent coprophagia. All rats were exposed to the same environmental conditions and were maintained on a proper diet and water ad libitum. The animals were randomly divided into seven groups, each consisting of eight animals: normal control group, acetic acid group, vehicle-treated group in which 1% carboxy methyl cellulose (CMC) was given. The drug-treated groups received sulphasalazine in a dose of 500 mg kg<sup>-1</sup> and Ginkgo-biloba in doses of 30, 60 and 120 mg kg<sup>-1</sup>, respectively. The drugs were suspended in 1% CMC and administered orally in a volume of 0.5 ml/100 g body weight. The doses were given once daily starting 48 h before the induction of colitis and continued for 5 consecutive days.

### 2.3. Induction of experimental colitis in rats

The animals were fasted for 24 h with access to water ad libitum before induction of colitis. Induction of colitis was performed using a modification of the method described by Millar et al. [20]. Each rat was sedated by an intraperitoneal injection of phenobarbitone (35 mg kg<sup>-1</sup>). Two milliliters of acetic acid (3%, v/v, in 9% saline) were infused for 30 s using a polyethylene tube (2 mm in diameter) which was inserted through the rectum into the colon to a distance of 8 cm. The acetic acid was retained in the colon for 30 s after which the fluid was withdrawn. Following completion of the experiment, rats were killed using ether anesthesia and colonic biopsies were taken for macroscopic scoring, histopathological examination and biochemical studies.

### 2.4. Assessment of colitis

#### 2.4.1. Macroscopic scoring

At post-mortem laparotomy, 6 cm of colon extending proximally for 2 cm above the anal orifice was removed. The tissue was first split longitudinally, pinned out onto card, and the macroscopic appearance of the colonic mucosa was scored by an independent observer according to a scale ranging from 0 to 4 as follows:

- 0 = No macroscopic changes
- 1 = Mucosal erythema only
- 2 = Mild mucosal oedema, slight bleeding or small erosions
- 3 = Moderate oedema, bleeding ulcers or erosions
- 4 = Severe ulceration, erosions, oedema and tissue necrosis [20]

#### 2.4.2. Histopathological study

Full thickness biopsy specimens were fixed in 10% formal saline prior to wax embedding, sectioning and staining with haematoxylin and eosin for histological evaluation of colonic damage by light microscopy.

### 2.5. Biochemical study

Colonic samples were stored immediately at -20 °C till analysis. Tissue samples were homogenized in 1 ml of 10 mmol/l Tris-HCl buffer of pH 7.1 and homogenate was used for the measurement of IL-1 $\beta$ , TNF- $\alpha$ , myeloperoxidase (MPO) activity, and reduced glutathione content (GSH).

### 2.6. Determination of myeloperoxidase activity

MPO activity had been used as index of leukocyte adhesion and accumulation in several tissues including the intestine. The principle of the method depends on release of MPO enzyme in the homogenate of the colonic tissue used. Its level was detected using 0.3 mmol of H<sub>2</sub>O<sub>2</sub> as a substrate. A unit of MPO activity is defined as that converting 1  $\mu$ mol of H<sub>2</sub>O<sub>2</sub> to water in 1 min at 25 °C [21]. In brief, segments of the distal colon (0.5 g) were homogenized in 10 vol. of 50 mM sodium phosphate buffer (pH 7.4) in an ice-bath using polytron homogenizer (50 mg tissue ml<sup>-1</sup>). The pellet (containing 95% of the total tissue MPO activity) was resuspended in an

equal volume of potassium phosphate buffer (pH 6). Another centrifugation step for a period of 20 min at  $16,000 \times g$  was done. The resultant supernatant was used for MPO assay using tetramethylbenzidine (TMB). The activity of MPO was measured using Jenway 6505 spectrophotometer at 655 nm.

### 2.7. Measurement of interleukin-1 $\beta$ production

The mucosal level of this cytokine was assayed using a commercially available IL-1 $\beta$  enzyme-immunometric assay (EIA) kit (Titerzyme<sup>®</sup> EIA rat interleukin-1 $\beta$ , assay Designs). Briefly, colonic mucosal samples kept at  $-70^\circ\text{C}$  were weighed and homogenized, after thawing in 10 vol. of the assay buffer. They were centrifuged at 3800 rpm for 20 min, at  $4^\circ\text{C}$ . Then 100  $\mu\text{l}$  of the supernatant 100  $\mu\text{l}$  of standard and assay buffer were added to the wells of a microtiter plate with an immobilized polyclonal antibody to rat IL-1 $\beta$ . After incubation at  $37^\circ\text{C}$  for 1 h, the excess sample or standard were washed out and a monoclonal antibody to rat IL-1 $\beta$  coupled to horseradish peroxidase was added. This labeled antibody binds to the rat IL-1 $\beta$  captured on the plate. After a short incubation, the excess labeled antibody was washed out and substrate was added. The substrate reacts with the labeled antibody bound to the rat interleukin-1 $\beta$  captured on the plate. The colour generated with the substrate was read at 450 nm in a microplate reader (Labysistem Multiskan EX) and was directly proportional to the concentration of rat IL-1 $\beta$  in either standard or sample. The interleukin-1 $\beta$  content is expressed as interleukin-1 $\beta$ /mg tissue [22].

### 2.8. Measurement of tumor necrosis factor- $\alpha$

TNF $\alpha$  was determined according to the method of Reinecker et al. [23]. Colonic samples were immediately weighed, minced on an ice-cold plate, suspended in a tube with 10 mmol/l sodium phosphate buffer (pH 7.4) (1:5, w/v). The tubes were placed in a shaking water bath ( $37^\circ\text{C}$ ) for 20 min and centrifuged at  $9000 \times g$  for 30 s at  $4^\circ\text{C}$ , and the supernatant was frozen at  $-80^\circ\text{C}$  until assay. TNF- $\alpha$  was quantified by enzyme-linked immunoabsorbent assay (Amersham Pharmacia Biotech, Little Chalfont, UK) and the results were expressed as picograms per gram of wet tissue.

### 2.9. Determination of reduced glutathione

Reduced glutathione was determined as previously described by Owens and Belcher [24] based on the reaction of 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) with the GSH present. The absorbance was measured at 412 nm in a Shimadzu double beam spectrophotometer (UV 200S). The amount of glutathione present in the sample was calculated using a standard solution of GSH containing 1 mg of GSH/1 ml of 3% metaphosphoric acid. The increase in the extinction at 412 nm is proportional to the amount of GSH present.

### 2.10. Statistical analysis

All data are expressed as mean  $\pm$  standard error of the mean (S.E.M.) for eight rats per experimental group. Statistical analy-

sis was performed with SPSS 10.0 statistical software. One-way analysis of variance (ANOVA) was used to compare the mean values of quantitative variables among the groups. Duncan's multiple range tests was used to identify the significance of pair-wise comparisons of mean values among the groups.

## 3. Results

### 3.1. Macroscopic results

The acetic acid treatment induced severe macroscopic inflammation in the colon 24 h after rectal administration, as assessed by the colonic damage score. Treatment with sulphasalazine significantly reduced the severity of the gross lesion score. On the other hand, Ginkgo biloba significantly reduced the intensity of inflammation in a dose-dependent manner as shown in (Table 1).

### 3.2. Microscopic results

The histopathological features of untreated rats included transmural necrosis, oedema and diffuse inflammatory cell infiltration in the mucosa. There was focal ulceration of the colonic mucosa extending through the muscularis mucosa, desquamated areas and loss of the epithelium. The architecture of the crypts was distorted and the lamina propria was thickened in peripheral areas of distorted crypts especially in basal areas. An infiltrate consisting of mixed inflammatory cells was observed (Fig. 1B). Treatment of rats with sulphasalazine (Fig. 1C) or Ginkgo biloba (Fig. 1D, E and F) significantly attenuated the extent and severity of the histological signs of cell damage, which was more obvious with Ginkgo biloba at the dose of  $120 \text{ mg kg}^{-1}$  (Fig. 1F). Histological studies confirmed the intestinal anti-inflammatory effect exerted by the two drugs used.

### 3.3. Myeloperoxidase activity

Tissue MPO activity showed a statistically significant difference among the groups tested ( $P < 0.001$ ). By performing pair-wise comparisons among the groups, we can infer that, the mean value of acetic acid control is significantly increased

Table 1  
Effects of different doses of Ginkgo-biloba (30, 60 and  $120 \text{ mg kg}^{-1}$ ) on gross lesion score of acetic acid-induced colitis in rats

Group	Treatment	Dose ( $\text{mg kg}^{-1}$ )	Gross lesion	% Reduction
1	Normal saline	–	$0.00 \pm 0.00$	–
2	Acetic acid	–	$3.83 \pm 0.17$	–
3	1% CMC	–	$3.75 \pm 0.16$	–
4	Sulphasalazine	500	$1.63 \pm 0.18^{\#}$	57 $^{\#}$
5	Ginkgo biloba	30	$1.67 \pm 0.2^{\#}$	56 $^{\#}$
6	Ginkgo biloba	60	$1.50 \pm 0.19^{\#}$	61 $^{\#}$
7	Ginkgo biloba	120	$1.25 \pm 0.16^{\#}$	67 $^{\#}$

Values are expressed as mean  $\pm$  S.E.M. of eight animals per group. The percentage reduction indicates the reduction of colonic lesion as compared to acetic acid group.

$^{\#} P < 0.001$  in comparison to acetic acid group.

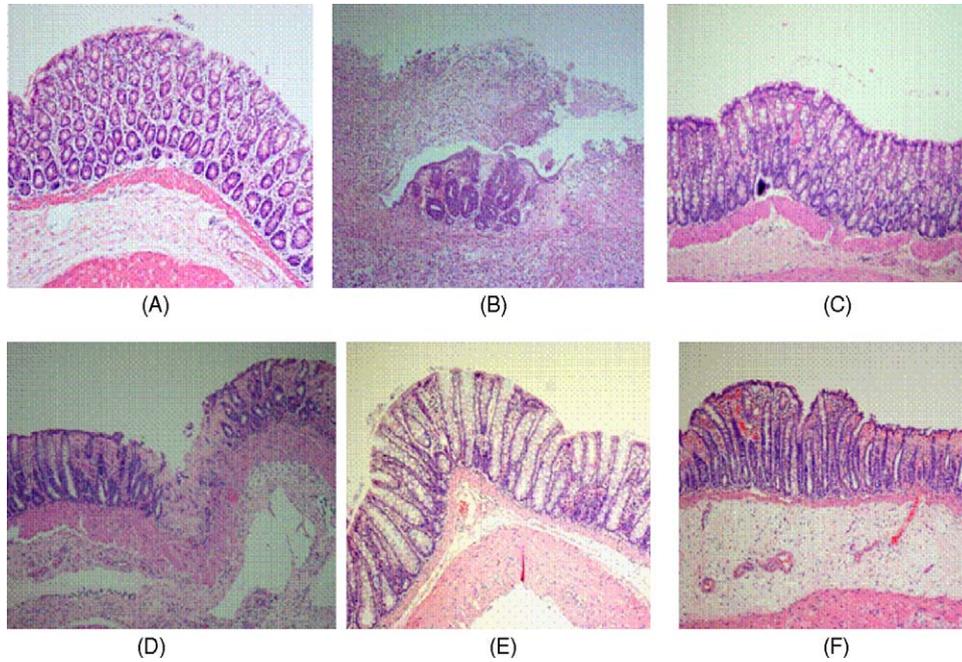


Fig. 1. (A) Normal control group. Photomicrograph of the haematoxylin and eosin stained section of rat colon showed intact epithelial surface;  $\times 100$ . (B) Acetic acid-treated animals, the colonic mucosa showed massive necrotic destruction of epithelium, submucosal oedema, haemorrhages and inflammatory cellular infiltration;  $\times 100$ . (C) Sulphasalazine-treated group. (C, D and F) Ginkgo biloba-treated groups, treatment with Ginkgo biloba showed a dose-dependent protective effect against acetic acid-induced damage; small doses of Ginkgo biloba ( $30$  and  $60$   $\text{mg kg}^{-1}$ ) produced mild and moderate protection (D and E, respectively) while complete protection (F) was observed with the highest dose of Ginkgo biloba ( $120$   $\text{mg kg}^{-1}$ );  $\times 100$ . Drugs were given orally once daily for 2 days before induction of colitis and continued for 5 consecutive days after induction of colitis.

( $P < 0.001$ ) as compared to the normal control group. Treatment with sulphasalazine ( $500$   $\text{mg kg}^{-1}$ , p.o.) or various doses of Ginkgo biloba ( $30$ ,  $60$  and  $120$   $\text{mg kg}^{-1}$ ) significantly reduced colonic MPO activity. The reduction was most significant at the dose of  $120$   $\text{mg kg}^{-1}$  (Fig. 2).

### 3.4. Interleukin-1 $\beta$ concentrations

Fig. 3 shows that the colonic inflammation induced by acetic acid significantly increased IL-1 $\beta$  level as compared to the normal control group ( $P < 0.001$ ). Administration of sulphasalazine ( $500$   $\text{mg kg}^{-1}$ , p.o.) or Ginkgo biloba ( $30$ ,  $60$  and  $120$   $\text{mg kg}^{-1}$ ,

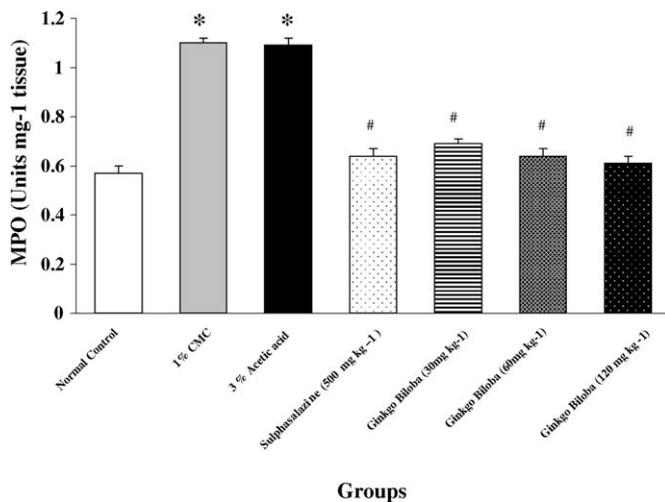


Fig. 2. Effects of different doses of Ginkgo biloba ( $30$ ,  $60$  and  $120$   $\text{mg kg}^{-1}$ ) on colonic myeloperoxidase (MPO) levels in acetic acid-induced colitis in rats. Results are expressed as mean  $\pm$  S.E.M. of eight observations. The vehicles or drugs were administered orally once daily for 2 days before induction of colitis and continued for 5 consecutive days after induction of colitis. \* $P < 0.001$  as compared to the saline control group. # $P < 0.001$  as compared to the acetic acid group.

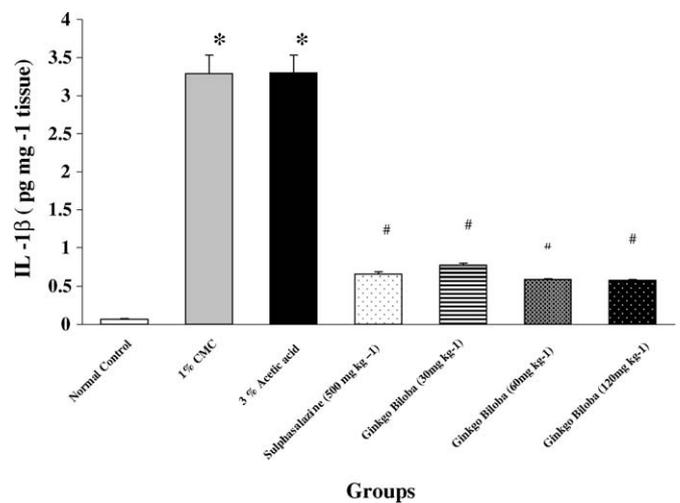


Fig. 3. Effects of different doses of Ginkgo biloba ( $30$ ,  $60$  and  $120$   $\text{mg kg}^{-1}$ ) on colonic interleukin-1 $\beta$  (IL-1 $\beta$ ) levels in acetic acid-induced colitis in rats. Results are expressed as mean  $\pm$  S.E.M. of eight observations. The vehicle or drugs were administered orally once daily for 2 days before induction of colitis and for 5 consecutive days after induction of colitis. \* $P < 0.001$  as compared to the saline control group. # $P < 0.001$  as compared to the acetic acid group.

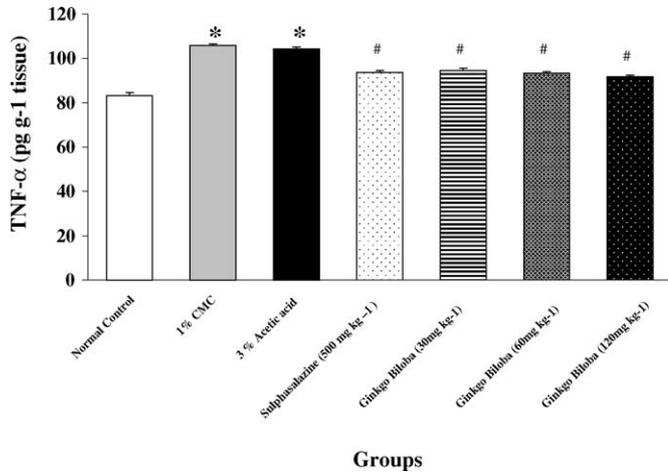


Fig. 4. Effects of different doses of Ginkgo biloba (30, 60 and 120 mg kg<sup>-1</sup>) on colonic tumor necrosis factor alpha levels (TNF- $\alpha$ ) in acetic acid-induced colitis in rats. Results are expressed as mean  $\pm$  S.E.M. of eight observations. The vehicle or drugs were administered orally once daily for 2 days before induction of colitis and for 5 consecutive days after induction of colitis. \* $P < 0.001$  as compared to the saline control group. # $P < 0.001$  as compared to the acetic acid group.

p.o.) resulted in a significant reduction in IL-1 $\beta$  in comparison with the acetic acid control group ( $P < 0.001$ ). Using pair-wise comparisons among the groups, Ginkgo biloba in a dose of 120 mg kg<sup>-1</sup> was the most potent at reducing IL-1 $\beta$  level.

### 3.5. Tumor necrosis factor- $\alpha$ concentrations

Fig. 4 demonstrates a significant increase in TNF- $\alpha$  activity in the inflamed colon at 24 h after acetic acid administration in comparison with normal control rats ( $P < 0.001$ ). Administration of either sulphasalazine (500 mg kg<sup>-1</sup>, p.o.) or Ginkgo biloba (30, 60 and 120 mg kg<sup>-1</sup>, p.o.) to rats resulted in a sig-

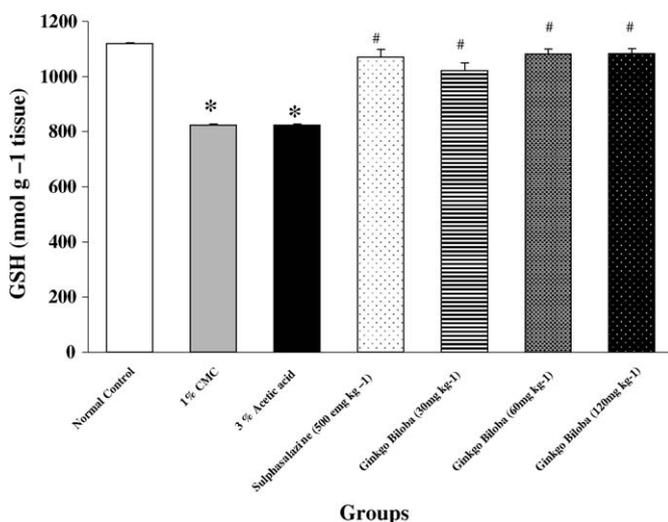


Fig. 5. Effects of different doses of Ginkgo biloba (30, 60 and 120 mg kg<sup>-1</sup>) on colonic reduced glutathione (GSH) content in acetic acid-induced colitis in rats. Results are expressed as mean  $\pm$  S.E.M. of eight observations. The vehicle or drugs were administered orally once daily for 2 days before induction of colitis and for 5 consecutive days after induction of colitis. \* $P < 0.001$  as compared to the saline control group. # $P < 0.001$  as compared to the acetic acid group.

nificant reduction in colonic TNF- $\alpha$  level. Maximum reduction was observed with Ginkgo biloba at a dose of 120 mg kg<sup>-1</sup>.

### 3.6. Reduced glutathione concentrations

Fig. 5 illustrates that mucosal GSH concentration was significantly decreased after induction of colitis as compared to the normal control group ( $P < 0.001$ ). After treatment with sulphasalazine or Ginkgo biloba in the same previously mentioned doses, there was a significant increase in the GSH concentration. The increase was substantially higher with Ginkgo-biloba at a dose of 120 mg kg<sup>-1</sup>.

## 4. Discussion

The present study demonstrated that treatment of rats with Ginkgo biloba reduced the inflammation and the acute colonic damage induced by acetic acid in a dose-dependent manner as verified by macroscopic, histological and biochemical data.

During the course of inflammatory bowel disease and experimental colitis, some proinflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$  are released and exacerbate tissue damage [25]. Our results showed an elevation in the levels of both TNF- $\alpha$  and IL-1 $\beta$  following the instillation of acetic acid in the colon of rats. Cytokines such as TNF- $\alpha$  and IL-1 $\beta$  when secreted by immunocytes in the inflamed intestine, can profoundly affect the activation state of mesenchymal cells, thereby amplifying the inflammatory response and probably contributing to fibrosis, one of the most important complications of inflammatory bowel disease. IL-1 $\beta$  and TNF- $\alpha$  stimulate proliferation of intestinal smooth muscle cells and fibroblasts and induce synthesis of IL-1 $\beta$ , IL-6, IL-8 and prostaglandin E<sub>2</sub> by these cells [26]. Both of these cytokines produce epithelial cell necrosis, oedema, neutrophil infiltration and, global cell depletion. It had been reported that blocking of the action of endogenous interleukin-1 $\beta$  and TNF- $\alpha$  attenuates acute and chronic experimental colitis and its systemic complications [9,27]. IL-1 $\beta$  stimulates anion secretion by epithelial cells indirectly through the liberation of prostaglandins. Also IL-1 $\beta$  augments hydrogen peroxide, bradykinin, and histamine-induced epithelial chloride secretion. Thus, IL-1 $\beta$  appears to be a primary stimulator of tissue damage and diarrhoea, the latter being a major symptom of intestinal inflammation [28]. Our results showed a significant reduction in the levels of these cytokines which may be explained by inhibition of their synthesis, production and release or inhibition of their biological activity. A similar explanation had been afforded by others [29,30].

The present study showed a significant increase in myeloperoxidase activity in the acetic acid group. This provides a quantitative measure of disease severity and a method of assessing drug efficacy in animal models of intestinal inflammation [31]. Measurement of MPO activity has been used as an indicator of neutrophil influx into inflamed gastrointestinal tissue [32]. Several investigators have demonstrated increased neutrophil infiltration in inflammatory mucosa [33,34]. Such an infiltration might be regarded as a trigger of free radicals release which may exert toxic effects on fatty acid residues in mem-

brane lipids. Increase in reactive oxygen species production and impaired antioxidant defense mechanisms are postulated to be causative factors in inflammatory diseases [35]. In the present investigation, Ginkgo biloba attenuated mucosal damage and subsequently reduced myeloperoxidase activity in colonic tissues. This protective effect may be attributed to the ability of Ginkgo biloba to reduce neutrophil infiltration in inflamed colonic tissue. Similar results had been reported by [36]. Further support to our results comes from the finding of Princemall et al. [37] who had demonstrated an inhibitory effects of Ginkgo biloba extract on myeloperoxidase activity and reactive oxygen species production from human leukocytes induced by phorbol myristate. Such inhibitory effects on neutrophils might result in reduced lipid peroxidation. The capability of Ginkgo biloba to scavenge superoxide anion, hydroxyl radicals and oxo-ferry radicals' species has been shown in several biological free radicals injury models [38–40].

The present study showed a significant reduction in GSH levels in acetic acid group. Similar results were observed by Sido et al. [41] and Millar et al. [20] who used the same model of colitis to test the antioxidant potential of 5-aminosalicylic acid. Furthermore, using trinitrobenzene sulphonic acid as a model of experimental colitis, Grisham et al. [42] and Barret [43] had shown a marked reduction in GSH levels. The reduction in the levels of GSH may be due to the liberation of oxygen-derived free radicals. Treatment of rats with Ginkgo biloba for 5 days resulted in an increase in colonic GSH levels. This may be explained by the radical scavenging capacity of Ginkgo biloba. Hibatallah et al. [44] attributed the protective effect of Ginkgo biloba to its scavenging effect on hydroxyl radical and superoxide anions. Other investigators have demonstrated that Ginkgo biloba stops lipoperoxidation by quenching the peroxy radical [45,46].

In this study, the development of acetic acid-induced colitis was studied in relation to macroscopic features of inflamed tissue (macroscopic damage score), which are used to quantify the severity of inflammation. The extent of colonic damage is dependent on initial concentration instilled into the rat colon [47]. In this study, an inflammatory response characterized by mucosal ulceration and heavy neutrophil infiltration of the mucosa and submucosa. Treatment with Ginkgo biloba resulted in attenuation of tissue damage and reduction in cell infiltration.

In conclusion, the present study indicate that Ginkgo biloba may be useful in the treatment of ulcerative colitis or other inflammatory diseases, especially if cytokines or free radicals is a part of its pathophysiology.

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