Chapter-V

Glycyrrhizic acid modulates 1,2-dimethylhydrazine-induced aberrant crypt foci and mucin depleted foci via regulating the hyperproliferation, inflammation, angiogenesis and apoptosis.
1. Background

Colon cancer is one of the most common, best-understood neoplasms from a genetic point of view, yet it remains the leading cause of cancer-related mortality in men and women (Brien et al. 2007; Jemal et al. 2009). Colon carcinogenesis is a multistep process and it emanate from a series of histopathological and molecular alterations (Janne and Mayer 2000). It thought to arise by the accretion of genetic alterations involving a variety of oncogenes and tumor suppressor genes that transform normal colonic epithelium into an invasive carcinoma (Janne and Mayer 2000; Khan et al. 2011; Mori et al. 2005).

About two millennia ago, Galen was the first who reported causal relationship between inflammation and cancer and later in the 19th century Rudolf Virchow had revealed that the tumors emanate from the area of chronic inflammation (Greten et al. 2004). Among the panoply of inflammatory mediators, NF-κB and TNF-α are the key factors involved in cancer-related inflammation (Mantovani et al. 2008). In inflammatory cells as well as in cells at risk of transformation by carcinogens, NF-κB mediates the transactivation of genes encoding inflammatory cytokines (viz., TNF-α), anti-apoptotic factors (viz., BCL-2), cyclooxygenase-2 (COX2), inducible nitric oxide synthase (iNOS) and angiogenic factors (viz., VEGF), (Orlowski et al. 2002; Mantovani et al. 2008). Mast cells play an important role in the initiation of inflammation. Mast cells have also been reported as being an essential hematopoietic component of the development of adenomatous polyps (Gounaris et al. 2007). Increased mast cell numbers have also been observed in patients with ulcerative colitis and Crohn’s disease, both of which are risk factors in colon cancer susceptibility (Andoh et al. 2006; He 2004). Recent experiments indicate that mast cell infiltration can enhance carcinogenesis (Theoharides and Koutsilieris 1997; Dimitriadou and Conti 2004) and they have also long been known to drive angiogenesis and
tumour growth (Soucek et al. 2007). In a 1,2-dimethylhydrazine-induced intestinal tumor model, the incidence of intestinal cancer was significantly reduced in mast cell–deficient KitW/KitW-v mice (Wedemeyer and Galli 2005). Although the mechanisms by which mast cells contribute to carcinogenesis are not understood. Mast cells are the only tissue-resident cells with granules containing preformed tumor necrosis factor-α (TNF-α), and releasing this cytokine from mast cells is important for the initiation of an inflammatory response (Galli et al. 2005). TNF-α is a pro-inflammatory cytokine that creates tumor microenvironment fostering tumor development by induction of tumor promoting cytokines, release of MMPs and pro-angiogenic activity (Coussens and Werb 2002). Recently, it has been reported that the expression of inflammatory markers viz., COX-2, and i-NOS are also enhanced together with orchestration of intracolonic infiltration of macrophage in MDF similarly as in colonic tumors. This might be due to reduced expression of MUC2 in MDF which consequently leads to the focal loss of the protective mucous layer. Thus, MDF are more exposed to toxicant present in the colon which ultimately results in the activation of inflammation in MDF. It has also been reported that inflammation provides a nidus for the development of MDF and colonic tumors, further supporting the concept that MDF are early preneoplastic lesions (Femia et al. 2009).

There is unequivocal evidence that chemoprevention is a pragmatic approach to inhibit or suppress the colon carcinogenesis or the development of ACF and MDF into adenoma or adenocarcinoma. ACF and MDF are being exploited as short-term bioassays to assess the chemopreventive potential of natural products against colon carcinogenesis (Wargovich et al. 2000). Thus, inhibiting or suppressing the development of ACF and MDF by natural products may be able to dampen the subsequent progression to colon cancer.
Chapter-V

Glycyrrhizic acid modulates...

Glycyrrhizic acid is a natural and major pentacyclic triterpenoid glycoside of licorice roots extracts (Zhang et al. 2009). It has several pharmacological and biological properties such as anti-inflammatory (Rao et al. 1994; Mollica et al. 2007; Davis and Morris 1991; Suzuki et al. 1983; Arase et al. 1997), anti-viral (Pompei et al. 1979; Curreli et al. 2005) and anti-cancer (Shiota et al. 1999; Agarwal et al. 1991; Yasukawa et al. 1988). It also induces apoptosis in several cancer cell lines such as human hepatoma (HLE), promyelotic leukemia (HL-60) and stomach cancer (KATO III) and prostate cancer cell lines (DU-145 and LNCaP) (Hibasami et al. 2005; Hibasami et al. 2006; Thirugnanam et al. 2008). Glycyrrhizic acid is commonly used in Japan as a therapeutic agent for the control and treatment of chronic viral hepatitis (Suzuki et al. 1983; Arase et al. 1997; van Rossum et al. 1999).

In the light of above facts, we designed this study to investigate the protective effects of glycyrrhizic acid against DMH-induced ACF and MDF and its role in regulating the hyperproliferation, inflammation, angiogenesis and apoptosis in the colon of Wistar rats.

2. Treatment Regimen

**Group 1 (Control):** Rats received basal diet along with distilled water (5ml/kg b.wt.).

**Group 2 (DMH):** Rats were administered with subcutaneous injection of DMH at the dose of 20mg/kg b.wt. once a week for first 5 weeks.

**Group 3 (DMH + GA) (I):** Rats were administered with DMH as in Gp 2 and also fed glycyrrhizic acid (15mg/kg b.wt. orally) every day for the first 5 weeks starting 1 week before carcinogen treatment (Initiation- I).
**Group 4 (DMH + GA) (PI):** Rats were administered with DMH as in Gp 2 and also fed glycyrrhizic acid (15mg/kg b.wt. orally) 2 days after the last injection of the carcinogen and continued till the end of the experiment (Post initiation-PI).

**Group 5 (Only GA):** Rats received basal diet + glycyrrhizic acid (15mg/kg b.wt. orally) dissolved in distilled water everyday throughout the experiment.
3. Results

3.1 Effect of glycyrrhizic acid and DMH on the development of ACF in colonic tissue
In DMH treated group (Group II), the number of ACF/colon is 86.33 ± 11 while supplementation with glycyrrhizic acid in Group III (82.67 ± 13) and IV (74.67 ± 8) were non-significantly reduced the number of ACF. Original magnification: 10x (Fig. 1 and 2)

3.2 Effect of glycyrrhizic acid and DMH on the development of MDF in colonic tissue
In DMH treated group (Group II), the number of MDF/colon is 4 ± 1.414 while supplementation with glycyrrhizic acid in Group III (3.33 ± 0.5164) reduced the number of MDF but at the non-significant level as compared to Group II. In Group IV, treatment with glycyrrhizic acid significantly reduced the number of MDF (2.5 ± 0.5477) (P< 0.05) as compared to Group II. Original magnification: 10x (Fig. 3 and 4)

3.3 Effect of glycyrrhizic acid and DMH on the mucin staining in colonic tissue
In DMH treated group (Group II), there is regional depletion of mucous layer (blue in color). Treatment with glycyrrhizic acid decreased the depletion of the mucous layer in Group IV as compared to Group II. There is no depletion of the mucous layer in colonic sections of Group I and Group V. (Fig. 5)

3.4 Effect of glycyrrhizic acid and DMH on the colonic sulphomucin and sialomucin
In DMH treated group, predominance of sialomucin (blue color) or shift from sulphomucin (brown color) to sialomucin (blue color) was observed. While treatment with glycyrrhizic acid markedly attenuated this shifting or there is predominance of sulphomucin as in control group. (Fig. 6)
3.5 Effect of glycyrrhizic acid and DMH on mast cell infiltration

In DMH treated group (Group II), there is infiltration of mast cells in the sub-mucosal layer below the lamina propria of the colonic section. Treatment with glycyrrhizic acid attenuated the infiltration of mast cells in Group III and IV as compared to Group II. There is no mast cells infiltration in colonic sections of Group I and Group V. (Fig. 7)

3.6 Effect of glycyrrhizic acid and DMH on the expression of Ki-67, NF-kB, COX-2, iNOS, VEGF, p53, caspase-9 and cleaved caspase-3 in colonic tissue

The colonic sections of DMH treated group (Group II) have more Ki-67, NF-kB, COX-2, iNOS and VEGF immunopositive staining while reduced P53, caspase-9 and cleaved caspase-3 immunopositive staining in DMH treated group (arrows) as indicated by brown colour as compared to control group (Group I). Treatment of glycyrrhizic acid reduced the immunostaining of Ki-67, NF-kB, COX-2, iNOS and VEGF while enhanced the immunostaining of p53, caspase-9 and cleaved caspase-3 in Group III and IV as compared to Group II. However, there were no significant differences in the immunostaining of all proteins in Group V as compared to Group I. For immunohistochemical analyses, brown colour indicates specific immunostaining of Ki-67, NF-kB, COX-2, iNOS, VEGF, p53, caspase-9 and cleaved caspase-3, and light blue colour indicates haematoxylin staining. Original magnification: 40x. (Fig. 8, 10-13, 15-17)

3.7 Effect of glycyrrhizic acid and DMH on the expression of Connexin-43 in colonic tissue

The colonic sections of DMH treated group (Group II) have reduced expression of connexin-43 immunopositive staining (arrows) as indicated by green color as compared to control group
(Group I) while treatment with glycyrrhizic acid in Group III and IV attenuated the immunostaining of connexin-43 as compared to Group II. However, there were no significant differences in the immunostaining of connexin-43 in Group V as compared to Group I. For fluorescent immunohistochemical analyses, green color indicates specific immunostaining of connexin-43, and red colour indicates propidium iodide staining. Original magnification: 40x. (Fig. 14)

3.8 Effect of glycyrrhizic acid and DMH on the level of TNF-α in colonic tissue
In DMH treated (Group II), the level of TNF-α was found to be significantly (p<0.001) elevated as compared to control group (Group I). Treatment with glycyrrhizic acid significantly attenuated the level of TNF-α in Group III (p<0.01) and IV (p<0.001) as compared to Group II. There is no significant difference between the levels of TNF-α in Group V as compared to Group I. (Fig. 9)

3.9 Effect of glycyrrhizic acid and DMH on the colonic histoarchitecture
The H&E stained sections of control group showed normal histoarchitecture with mild inflammatory cells infiltration while DMH treated group (Group II) exhibited intense inflammatory cells infiltration, irregular glandular structure along with crypt ablation. In group III and IV, histological sections showed that treatment with glycyrrhizic acid showed protection against DMH induced mucosal damage with marked reduction in the inflammatory cells infiltration. Colonic sections of Group V (only glycyrrhizic acid treated group) displayed normal histology as similar to that of Group I (control group). (Fig. 18)
4. Discussion

To the best of our knowledge, this is the first report to demonstrate the potential of glycyrrhizic acid to suppress DMH-induced ACF and MDF. In this study, we have observed that treatment with glycyrrhizic acid suppresses the development of early markers of colon cancer i.e., ACF and MDF. These protective effects of glycyrrhizic acid may be associated with the regulation of hyperproliferation, inflammation, angiogenesis and apoptosis in the colon of Wistar rats. Glycyrrhizic acid supplementation reduced the number of ACF but not at the significant level in both the group i.e., initiation and post-initiation group as compared to DMH treated group. The number of MDF is reduced significantly in post-initiation group while non-significantly in initiation group as compared to DMH treated group. These findings indicate that glycyrrhizic acid effectively suppresses the early events of colon carcinogenesis in Wistar rats.

Mucins are high molecular weight, heavily glycosylated proteins secreted by epithelial cells of the colon which forms a protective mucous layer in the form of gel in intestinal lumen (Robbe et al. 2004; Specian and Oliver 1991). The loss of the mucous layer might induce inflammation in this region since this region is now more exposed to various noxious agents present in colonic lumen. Our results showed that there was marked mucin depletion (blue color) in DMH treated group as compared to control group while supplementation with glycyrrhizic acid attenuated the mucin depletion to some extent.

There are basically two types of mucin i.e., sulphomucin (brown color) and sialomucin (blue color). The normal human colorectal mucosa and the distal part of the rat colon predominantly secrete sulphomucin. Previous studies carried out in histological sections have shown that apparently normal colonic mucosa from patients with colon cancer and dysplastic foci observed in the distal colon of carcinogen-treated rats produce predominantly sialomucins instead of
sulphomucins as the normal mucosa (Filipe and Branfoot 1974; Filipe 1975; Wargovich et al. 1983; Sandforth et al. 1988). We observed only the distal part of the rat colons since the distal colon shows a pattern of mucus production similar to that of the normal human colorectal mucosa in which sulphomucin secretion predominates and since precancerous alterations in the human and distal rat colon are accompanied by a shift from sulphomucin to sialomucin secretion (Filipe and Branfoot 1974; Filipe 1975). In this study, there is predominance of sialomucin (blue color) in the colon of DMH-treated rats as compared to control rats which exhibited predominance of sulphomucin (brown color) while treatment with glycyrrhizic acid attenuated this shifting from sulphomucin to sialomucin.

Previous investigations have been shown that glycyrrhizic acid has anti-inflammatory property (Rao et al. 1994; Mollica et al. 2007; Davis and Morris 1991; Suzuki et al. 1983; Arase et al. 1997). Infiltration of mast cells is the sign of initiation of inflammation. In this study, it was observed that there is marked infiltration of mast cells in the sub-mucosal layer in DMH-treated group while there is no mast cells infiltration in control group. Treatment with glycyrrhizic acid markedly reduced the infiltration of mast cells within the sub-mucosal layer.

NF-kB activated in response to inflammation (Mantovani et al. 2008) and carcinogens (Karin and Greten 2005). Once activated, NF-kB transactivate the genes encoding TNF-α, COX-2, iNOS, and VEGF. Our findings showed that in DMH-treated group there is more NF-kB immunopositive staining as compared to control group while treatment with glycyrrhizic acid markedly attenuated the NF-kB immunopositive staining.

In the present investigation, it was also observed that the level of TNF-α was significantly increased in DMH-treated group as compared to control group while treatment with glycyrrhizic acid significantly attenuated the level of TNF-α.
In DMH-treated group, there is more immunopositive staining of other inflammatory mediators i.e., COX-2 and iNOS as compared to control group. While treatment with glycyrrhizic acid markedly attenuated the immunopositive staining of COX-2 and iNOS. Our results suggest that glycyrrhizic acid has strong anti-inflammatory potential and these results corroborated with previous reports.

In our study, it was observed that in DMH-treated group there is more VEGF immunopositive staining as compared to control group while treatment with glycyrrhizic acid markedly attenuated the VEGF immunopositive staining. These results exhibited that glycyrrhizic acid suppresses the expression of VEGF which is a marker of angiogenesis and thus suggest having anti-angiogenic effects.

In the previous studies, it has been observed that glycyrrhizic acid suppresses the hyperproliferative responses via down-regulation of the expression of proliferation marker i.e., proliferating cell nuclear antigen (PCNA) (Cherng et al. 2011). Ki-67 is also a cell proliferation marker which is a nuclear protein mainly express in proliferating cells (Scholzen and Gerdes 2000). In our study, it was observed that DMH-treated group has more Ki-67 immunopositive staining as compared to control group while treatment with glycyrrhizic acid significantly attenuated the Ki-67 immunopositive staining. These results further corroborated with the previous findings that glycyrrhizic acid suppresses the hyperproliferative responses in the colon of Wistar rats.

Connexin-43 is one of the transmembrane protein that form channels between adjacent cells known as gap junctions (Saez et al. 2003). Gap junctions allows direct exchange of small molecules like metabolites and second messengers and consequently biologic signalling between
cells which are critical for tissue development, cellular differentiation, cell proliferation and apoptosis (Kanczuga-Koda et al. 2006; Krutovskikh et al. 2002). Connexin-43 also acts as a colorectal cancer tumor suppressor and that the loss of connexin-43 expression during colorectal cancer development is associated with reduced patient survival. The expression of connexin-43 is commonly down-regulated in tumors, leading to loss of gap junctional intercellular communication (Sirnes et al. 2011). It has also been reported to regulate growth of colon cancer cells via inducing apoptosis (Sirnes et al. 2011). The p53 is a tumor suppressor protein and also acts as a transcription factor that regulates the transcription of genes involved in cell cycle, DNA repair and apoptosis (Riley et al. 2008). However, several lines of evidence indicate that the proapoptotic activity of p53 is independent of its function as a transcription factor (Schuler et al. 2000). p53 induces apoptosis via intrinsic pathway (mitochondrial pathway) by evoking cytochrome c release from the mitochondria that leads to the activation of Apaf-1 and caspase 9. Caspase-9 in turn ultimately leads to the activation of caspase-3 (Jin and Levine 2001).

In this study, it was observed that DMH-treated group has reduced immunopositive staining of connexin-43 as well as p53 as compared to control group which showed that there is reduced expression of connexin-43 and p53 while treatment with glycyrrhizic acid significantly attenuated the immunopositive staining of connexin-43 and p53. It was also observed that DMH-treated group has reduced immunopositive staining of caspase-9 and cleaved caspase-3 as compared to control group while treatment with glycyrrhizic acid significantly enhanced the immunopositive staining of caspase-9 and cleaved caspase-3. These results exhibited that treatment with glycyrrhizic acid induce apoptosis and these results corroborated with the previous reports which showed that glycyrrhizic acid induces apoptosis in several cancer cell lines (Hibasami et al 2005; Hibasami et al. 2006; Thirugnanam et al. 2008).
Histological findings revealed that control group showed normal histoarchitecture with mild infiltration of inflammatory cells as well as intact mucosal glandular structure while DMH-treated group exhibited massive infiltration of inflammatory cells in the lamina propria, distorted mucosal glandular architecture along with crypt ablation and crypt abscess formation. Treatment with glycyrrhizic acid strongly suppressed the infiltration of inflammatory cells in the mucosal layer, reduced the severity of submucosal edema, crypt abscess formation and crypt ablation induced by DMH in the colon of Wistar rats. Histological findings clearly revealed that glycyrrhizic acid has strong anti-inflammatory property. The above mentioned findings corroborated with the histological data which exhibited the protective effects of glycyrrhizic acid against DMH-induced colonic damage.

It can be concluded from the findings of the present study that glycyrrhizic acid has chemopreventive potential against DMH-induced colon carcinogenesis via suppressing the development of precancerous lesions i.e., ACF and MDF in the colon of Wistar rats. The precise mechanism of protective action of glycyrrhizic acid against DMH induced colonic damage is still unknown but the probable mechanism through the attenuation of hyperproliferation, inflammation, angiogenesis and apoptotic responses in the colon of Wistar rats. Further studies are warranted to elucidate the exact protective mechanism of glycyrrhizic acid.
Figure 1. Topographic view of Aberrant Crypt Foci (ACF) in a rat colon.

(A)  (B)

(C)  (D)

Figure 1. ACF in the rat colon showing round and elongated ACF (Fig. A, B, C and D) with different crypt multiplicities. The colons were opened, stained with methylene blue and observed on a glass slide. Original magnification: 10x.

Figure 2. Effects of glycyrrhizic acid and DMH on the Aberrant Crypt Foci (ACF).

<table>
<thead>
<tr>
<th>DMH Gp</th>
<th>I Gp (GA+ DMH)</th>
<th>PI Gp (DMH+GA)</th>
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<tbody>
<tr>
<td>ACF / Colon</td>
<td>NS</td>
<td>NS</td>
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Figure 2. Effects of DMH and glycyrrhizic acid on incidence of ACF per rat colon. Values are expressed as Mean ± SD. Glycyrrhizic acid non-significantly suppressed the development of ACF in Group 3 and 4 as compared to DMH treated group (Group 2). NS- Non-significant.
Figure 3. Topographic view of Mucin Depleted Foci (MDF) in a rat colon.

(A)  
(B)  
(C)  
(D)  

Figure 3. MDF in the rat colon showing depleted mucin (Fig. B, C and D). The colons were opened, stained with high iron diamine (HID) and alcian blue (AB). Original magnification: 10x.

Figure 4. Effects of glycyrrhizic acid and DMH on the Mucin Depleted Foci (MDF).

Figure 4. Effects of DMH and glycyrrhizic acid on incidence of MDF per rat colon. Values are expressed as Mean ± SD. Glycyrrhizic acid non-significantly suppressed the development of ACF in Group 3 and 4 as compared to DMH treated group (Group 2). NS- Non-significant.
Figure 5. Effects of glycyrrhizic acid and DMH on the mucin staining

Figure 5. Photomicrographs of colonic sections depicting (A) Vehicle treated control group (Group I), (B) DMH treated group (20mg/kg b. wt.) (Group II), (C) DMH + GA (15 mg/kg b.wt.) + DMH (Initiation group or Group III), (D) DMH + GA (15 mg/kg b.wt.) + DMH (Post-initiation group or Group III), (E) Only GA (15 mg/kg b.wt.) (Group V). In DMH treated group (Group II), there is regional depletion of mucin in the form of mucous layer (blue in color). Treatment with glycyrrhizic acid decreased the depletion of the mucous layer in Group IV as compared to Group II. No effects of glycyrrhizic acid on mucous layer in Group III as compared to Group II. There is no depletion of the mucous layer in colonic sections of Group I and Group V. Insets at the right panel show a magnified view (40x magnifications) of the insets showed at the left panel (10x magnifications).
Figure 6. Effects of glycyrrhizic acid and DMH on the sulphomucin and sialomucin.

Figure 6. Photomicrographs of colonic sections depicting (A) Vehicle treated control group (Group I), (B) DMH treated group (20mg/kg b. wt.) (Group II), (C) DMH + GA (15 mg/kg b.wt.) + DMH (Initiation group or Group III), (D) DMH + GA (15 mg/kg b.wt.) + DMH (Post-initiation group or Group III), (E) Only GA (15 mg/kg b.wt.) (Group V). In DMH treated group, predominance of sialomucin (blue color) or shift from sulphomucin (brown color) to sialomucin (blue color) was observed. While treatment with glycyrrhizic acid markedly attenuated this shifting or there is predominance of sulphomucin as in control group. Insets at the right panel show a magnified view (40x magnifications) of the insets showed at the left panel (10x magnifications).
Figure 7. Effects of glycyrrhizic acid and DMH on the colonic mast cells infiltration.

Figure 7. Photomicrographs of colonic sections depicting (A) Vehicle treated control group (Group I), (B) DMH treated group (20mg/kg b. wt.) (Group II), (C) DMH + GA (15 mg/kg b.wt.) (Initiation group or Group III), (D) DMH + GA (15 mg/kg b.wt.) (Post-initiation group or Group III), (E) Only GA (15 mg/kg b.wt.) (Group V). In DMH treated group (Group II), there is infiltration of mast cells in the sub-mucosal layer below the lamina propria of the colonic section. Pretreatment with glycyrrhizic acid attenuated the infiltration of mast cells in Group III and IV as compared to Group II. There is no mast cells infiltration in colonic sections of Group I and Group V. Insets at the right panel show a magnified view (40x magnifications) of the insets showed at the left panel (10x magnifications).
Figure 8. Immunohistochemical staining of NF-kB

Figure 8. Photomicrographs of colonic sections depicting (A) Vehicle treated control group (Group I), (B) DMH treated group (20mg/kg b. wt.) (Group II), (C) DMH + GA (15 mg/kg b.wt.) (Initiation group or Group III), (D) DMH + GA (15 mg/kg b.wt.) (Post-initiation group or Group III), (E) Only GA (15 mg/kg b.wt.) (Group V). For immunohistochemical analyses, brown colour indicates specific immunostaining of NF-kB and light blue colour indicates nuclear haematoxylin staining. The colonic section of DMH-treated group (Group II) has more NF-kB immunopositive staining as indicated by brown colour as compared to control group (Group I) while treatment with glycyrrhizic acid in Group III and IV reduced NF-kB immunostaining as compared to Group II. However there was no significant difference in the NF-kB immunostaining in Group V as compared to Group I. Original magnification: 40x.
Figure 9. Effects of glycyrrhizic acid and DMH on the TNF-α level.

Figure 9: Effect of glycyrrhizic acid against DMH on TNF-α level. (A) Vehicle treated control group (Group I), (B) DMH treated group (20mg/kg b. wt.) (Group II), (C) DMH + GA (15 mg/kg b.wt.) (Initiation group or Group III), (D) DMH + GA (15 mg/kg b.wt.) (Post-initiation group or Group III), (E) Only GA (15 mg/kg b.wt.) (Group V). In DMH-treated group, the level of TNF-α was increased significantly (***p<0.001) as compared to control group. While treatment with glycyrrhizic acid significantly attenuated the level of TNF-α in Group III (##p<0.01) and IV (###p<0.001) as compared to Group II. There was no significant difference in the level of TNF-α in Group V as compared to Group I.
Figure 10. Photomicrographs of colonic sections depicting (A) Vehicle treated control group (Group I), (B) DMH treated group (20mg/kg b.wt.) (Group II), (C) DMH + GA (15 mg/kg b.wt.) (Initiation group or Group III), (D) DMH + GA (15 mg/kg b.wt.) (Post-initiation group or Group III), (E) Only GA (15 mg/kg b.wt.) (Group V). For immunohistochemical analyses, brown colour indicates specific immunostaining of COX-2 and light blue colour indicates nuclear haematoxylin staining. The colonic section of DMH-treated group (Group II) has more COX-2 immunopositive staining as indicated by brown colour as compared to control group (Group I) while treatment with glycyrrhizic acid in Group III and IV reduced COX-2 immunostaining as compared to Group II. However there was no significant difference in the COX-2 immunostaining in Group V as compared to Group I. Original magnification: 40x.
Figure 11. Photomicrographs of colonic sections depicting (A) Vehicle treated control group (Group I), (B) DMH treated group (20mg/kg b. wt.) (Group II), (C) DMH + GA (15 mg/kg b.wt.) (Initiation group or Group III), (D) DMH + GA (15 mg/kg b.wt.) (Post-initiation group or Group III), (E) Only GA (15 mg/kg b.wt.) (Group V). For immunohistochemical analyses, brown colour indicates specific immunostaining of iNOS and light blue colour indicates nuclear haematoxylin staining. The colonic section of DMH-treated group (Group II) has more iNOS immunopositive staining as indicated by brown colour as compared to control group (Group I) while treatment with glycyrrhizic acid in Group III and IV reduced iNOS immunostaining as compared to Group II. However there was no significant difference in the iNOS immunostaining in Group V as compared to Group I. Original magnification: 40x.
Figure 12. Immunohistochemical staining of VEGF

(A) Vehicle treated control group (Group I), (B) DMH treated group (20mg/kg b. wt.) (Group II), (C) DMH + GA (15 mg/kg b.wt.) (Initiation group or Group III), (D) DMH + GA (15 mg/kg b.wt.) (Post-initiation group or Group III), (E) Only GA (15 mg/kg b.wt.) (Group V). For immunohistochemical analyses, brown colour indicates specific immunostaining of VEGF and light blue colour indicates nuclear haematoxylin staining. The colonic section of DMH-treated group (Group II) has more VEGF immunopositive staining as indicated by brown colour as compared to control group (Group I) while treatment with glycyrrhizic acid in Group III and IV reduced VEGF immunostaining as compared to Group II. However there was no significant difference in the VEGF immunostaining in Group V as compared to Group I. Original magnification: 40x.
Figure 13. Immunohistochemical staining of Ki-67

(A) Vehicle treated control group (Group I), (B) DMH treated group (20mg/kg b. wt.) (Group II), (C) DMH + GA (15 mg/kg b.wt.) (Initiation group or Group III), (D) DMH + GA (15 mg/kg b.wt.) (Post-initiation group or Group III), (E) Only GA (15 mg/kg b.wt.) (Group V). For immunohistochemical analyses, brown colour indicates specific immunostaining of Ki-67 and light blue colour indicates nuclear haematoxylin staining. The colonic section of DMH-treated group (Group II) has more Ki-67 immunopositive staining as indicated by brown colour as compared to control group (Group I) while treatment with glycyrrhizic acid in Group III and IV reduced Ki-67 immunostaining as compared to Group II. However there was no significant difference in the Ki-67 immunostaining in Group V as compared to Group I. Original magnification: 40x.
Figure 14. Fluorescent immunohistochemical staining of Connexin-43

(A) (B)

(C) (D)

(E)

Figure 14. Photomicrographs of colonic sections depicting (A) Vehicle treated control group (Group I), (B) DMH treated group (20mg/kg b. wt.) (Group II), (C) DMH + GA (15 mg/kg b.wt.) (Initiation group or Group III), (D) DMH + GA (15 mg/kg b.wt.) (Post-initiation group or Group III), (E) Only GA (15 mg/kg b.wt.) (Group V). For fluorescent immunohistochemical analyses, green colour indicates specific immunostaining of connexin-43 and red colour indicates nuclear propidium iodide staining. The colonic section of DMH treated group (Group II) has reduced connexin-43 immunopositive staining (arrows) as indicated by brown colour as compared to control group (Group I) while treatment with glycyrrhizic acid attenuated connexin-43 immunostaining in Group III and IV as compared to Group II. However, there was no significant difference in the connexin-43 immunostaining in Group V as compared to Group I. Original magnification: 40x.
Figure 15. Immunohistochemical staining of p53

Figure 15. Photomicrographs of colonic sections depicting (A) Vehicle treated control group (Group I), (B) DMH treated group (20mg/kg b. wt.) (Group II), (C) DMH + GA (15 mg/kg b.wt.) (Initiation group or Group III), (D) DMH + GA (15 mg/kg b.wt.) (Post-initiation group or Group III), (E) Only GA (15 mg/kg b.wt.) (Group V). For immunohistochemical analyses, brown colour indicates specific immunostaining of p53 and light blue colour indicates nuclear haematoxylin staining. The colonic section of DMH-treated group (Group II) has reduced immunopositive staining of p53 as indicated by brown colour as compared to control group (Group I) while treatment of glycyrrhizic acid in Group III and IV enhanced the immunopositive staining of p53 as compared to Group II. However there was no significant difference in the p53 immunostaining in Group V as compared to Group I. Original magnification: 40x.
Figure 16. Photomicrographs of colonic sections depicting (A) Vehicle treated control group (Group I), (B) DMH treated group (20mg/kg b. wt.) (Group II), (C) DMH + GA (15 mg/kg b.wt.) (Initiation group or Group III), (D) DMH + GA (15 mg/kg b.wt.) (Post-initiation group or Group III), (E) Only GA (15 mg/kg b.wt.) (Group V). For immunohistochemical analyses, brown colour indicates specific immunostaining of caspase-9 and light blue colour indicates nuclear haematoxylin staining. The colonic section of DMH-treated group (Group II) has reduced immunopositive staining of caspase-9 as indicated by brown colour as compared to control group (Group I) while treatment of glycyrrhizic acid in Group III and IV enhanced the immunopositive staining of caspase-9 as compared to Group II. However there was no significant difference in the caspase-9 immunostaining in Group V as compared to Group I. Original magnification: 40x.
Figure 17. Immunohistochemical staining of Cleaved Caspase-3

(A) Vehicle treated control group (Group I), (B) DMH treated group (20mg/kg b. wt.) (Group II), (C) DMH + GA (15 mg/kg b.wt.) (Initiation group or Group III), (D) DMH + GA (15 mg/kg b.wt.) (Post-initiation group or Group III), (E) Only GA (15 mg/kg b.wt.) (Group V). For immunohistochemical analyses, brown colour indicates specific immunostaining of cleaved caspase-3 and light blue colour indicates nuclear haematoxylin staining. The colonic section of DMH-treated group (Group II) has reduced immunopositive staining of cleaved caspase-3 as indicated by brown colour as compared to control group (Group I) while treatment of glycyrrhizic acid in Group III and IV enhanced the immunopositive staining of cleaved caspase-3 as compared to Group II. However there was no significant difference in the cleaved caspase-3 immunostaining in Group V as compared to Group I. Original magnification: 40x.
Figure 18. Effects of glycyrrhizic acid and DMH on the colonic histoarchitecture

Figure 18. Photomicrographs of colonic sections depicting (A) Vehicle treated control group (Group I), (B) DMH treated group (20mg/kg b. wt.) (Group II), (C) DMH + GA (15 mg/kg b.wt.) (Initiation group or Group III), (D) DMH + GA (15 mg/kg b.wt.) (Post-initiation group or Group III), (E) Only GA (15 mg/kg b.wt.) (Group V). The histological sections of control group showed normal histoarchitecture while DMH treated group (Group II) exhibited intense inflammatory cells infiltration, irregular glandular structure along with crypt ablation. In group III and IV, histological sections showed that treatment with glycyrrhizic acid showed protection against DMH induced colonic damage. Colonic sections of Group V (only glycyrrhizic acid treated group) displayed normal histology as similar to that of Group I (control group). Insets at the right panel show a magnified view (40x magnifications) of the insets showed at the left panel (10x magnifications).