Chapter-VI

Chemoprevention of colon tumorigenesis by glycyrrhizic acid in Wistar rats
1. Background

The concept of prevention of cancer using naturally occurring substances is gaining attention of several investigators. A number of naturally occurring products from vegetables and herbs exert their chemopreventive properties against various diseases including carcinogenesis (Wattenberg 1979). Chemoprevention is defined as the use of natural products or synthetic agents to prevent, suppress or reverse the carcinogenesis process before it reaches to malignancy (Sporn et al. 1976). Therefore, chemoprevention has emerged as a pragmatic approach to inhibit or suppress the development of colon carcinogenesis. 1,2-dimethylhydrazine induced colon tumorigenesis are being exploited as a model to assess the chemopreventive potential of natural products against colon cancer. Thus, inhibiting or suppressing the development of colonic tumors by natural products may be able to suppress the subsequent progression from adenoma to invasive adenocarcinoma (Wargovich et al. 2000).

Glycyrrhizic acid (GA), the most studied active constituent of licorice, is a sweet-tasting compound and it is a natural pentacyclic triterpenoid glycoside of licorice roots extracts. In many countries like Japan, GA is used as a major therapeutic agent to treat chronic viral hepatitis (Suzuki et al. 1983; Arase et al. 1997; van Rossum et al. 1999). It is also known to have several others pharmacological and biological properties such as anti-inflammatory (Rao et al. 1994; Mollica et al. 2007; Zhang et al. 2009; Davis and Morris 1991), anti-ulcerous (Doll and Hill 1962), interferon (IFN)-inducing activity (Abe et al. 1982), antiviral (Pompei et al. 1979; Curreli et al. 2005; Suzuki et al. 1983; Arase et al. 1997; van Rossum et al. 1999; Cinatl et al. 2003) and anti-carcinogenesis (Shiota et al. 1999; Ikeda et al. 2006; Agarwat et al. 1991; Zhang et al. 2009; Watari et al. 1976; Nishino 1992) effects. GA 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).

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 is thought to be 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.

1,2-dimethylhydrazine (DMH) is a colon specific carcinogen and it has been widely used to induce colon cancer in rodents (Heitman et al. 1983). DMH undergoes metabolism mainly in the liver and to some extent also in the colon and the ultimate metabolite thus formed in the liver is delivered to the colon via, blood or bile, as glucoronide conjugates (Oravec et al. 1986; Sengottuvelan et al. 2006). In vivo transformation of DMH results in the formation of azomethane and N-oxidation of azomethane leads to the formation of azoxymethane. Further, hydroxylation of azoxymethane leads to the formation of methyazoxymethanol which is an unstable compound and readily yields highly reactive electrophilic methyldiazonium ion. The latter leads to the formation of methyl free radicals which forms adduct with DNA and ultimately lead to the development of colon cancer (Fiala 1977).

Inflammation and cancer are intricately related to each other and in the 19th century Rudolf Virchow had revealed that the tumors emanate from the area of chronic inflammation (Greten et al. 2004). Several epidemiological studies have shown that chronic inflammation predisposes
individuals to various types of cancer. Treatment with non-steroidal anti-inflammatory drugs reduces the incidence of, and the mortality that results from, several tumour types and it further supports the involvement of inflammation in cancer (Koehne and Dubois 2004; Flossmann and Rothwell 2007; Chan et al. 2007).

Apoptosis, one of the forms of programmed cell death, characterized by chromatin condensation, nuclear fragmentation, membrane blebbing, cytoskeletal rearrangement and cell shrinkage. It is involved in many physiological and pathological processes and helps to regulate tissue homeostasis by eliminating potentially deleterious cells (Khan et al. 2007). Reduced apoptosis is generally associated with tumorigenesis, so genes involved in the negative regulation of this process might have an oncogenic potential (e.g., Bcl-2, Survivin), whereas genes involved in the positive regulation of apoptosis might act as a tumor suppressor genes (e.g., Bax, Bak). There is now accumulating evidence that some pro-apoptotic genes, in combination with other genes, functions as tumor suppressor. In addition, it appears that in many cases negative regulators of apoptosis might act as oncogenes, but only in cooperation with other proteins apparently, proto-oncogenes.

In the light of above facts, we designed this study to investigate the chemopreventive potential of glycyrrhizic acid against DMH-induced colon tumorigenesis 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 15 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 15 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.

![Diagram of experimental design](image)

Figure: depicts the schematic representation of the experimental design
3. Results

3.1 Effects of DMH and glycyrrhizic acid on tumor incidence and multiplicity

Representative photomicrographs of morphological appearance of tumors are shown in Fig. 1. Most of the colonic tumors were developed in the distal colon of the Wistar rats. The colonic tumor incidence [(Number of tumor-bearing rats/total number of rats in each group) X 100] and tumor multiplicity (number of tumors/tumor bearing rats) in the treatment groups are summarized in Table 1. No tumors were observed in control animals (Group I) and only GA fed group (Group V). Significant inhibition occurred in the tumor incidence and multiplicity in the DMH+GA group (Group IV) as compared to DMH treated group (Group II) while in DMH+GA (Group III), there is inhibition occurred in the tumor incidence and multiplicity as compared to DMH treated group (Group II) but not at the significant level.

3.2 Effects of DMH and glycyrrhizic acid on colonic histopathological alteration

Representative photomicrograph of a hematoxylin and eosin stained colonic sections of control group showed normal histoarchitecture with mild inflammatory cells infiltration while DMH treated group (Group II) exhibited in situ adenocarcinoma with papillary pattern, dysplastic zone, marked cellular atypia, loss of goblet cell differentiation, nuclear pseudostratification, high nucleus to cytoplasmic ratio and massive inflammatory cells infiltration. In group III and IV, glycyrrhizic acid supplementation lessens the severity of DMH induced colonic tumors via reducing the infiltration of inflammatory cells within the colonic tumors. Colonic sections of Group V (only glycyrrhizic acid treated group) displayed normal histology as similar to that of Group I (control group). (Fig. 2)
3.3 Effect of glycyrrhizic acid and DMH on the colonic sulphomucin and sialomucin pattern

In DMH treated group, there is predominance of sialomucin (blue color) or shift from sulphomucin (brown color) to sialomucin (blue color) was observed within the colonic tumor sections. While treatment with glycyrrhizic acid attenuated this shifting to some extent as compares to DMH treated group. In Group I and Group V, there is predominance of sulphomucin and there is no difference in between Group I and Group V (brown color) (Fig. 3)

3.4 Effect of glycyrrhizic acid and DMH on mast cell infiltration

In DMH treated group (Group II), there is intense infiltration of mast cells in the sub-mucosal layer below the lamina propria within the colonic tumor 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. 4)

3.5 Effect of glycyrrhizic acid and DMH on the expression of Ki-67, PCNA, NF-kB, COX-2, iNOS, VEGF, p53 and cleaved caspase-3

The colonic sections of DMH treated group (Group II) have more Ki-67, PCNA, NF-kB, COX-2, iNOS and VEGF immunopositive staining within the tumor as well as in adjacent to tumor while reduced p53 and cleaved caspase-3 immunopositive staining within the tumor as well as in adjacent to tumor tissue as indicated by brown colour as compared to control group (Group I). Treatment with glycyrrhizic acid reduced the immunostaining of Ki-67, PCNA, NF-kB, COX-2, iNOS and VEGF while enhanced the immunostaining of p53 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, PCNA, NF-kB, COX-2, iNOS, VEGF, p53 and cleaved caspase-3, and light blue colour indicates haematoxylin staining. Original magnification: 40x. (Fig. 5-10, 12, 15)

3.6 Effect of glycyrrhizic acid and DMH on the expression of Connexin-43, Bcl-2 and Survivin in colonic tissue

The colonic sections of DMH treated group (Group II) have reduced connexin-43 immunopositive staining while enhanced Bcl-2 and survivin immunopositive staining within the colonic tumor as well in adjacent to tumor tissue as indicated by green color as compared to control group (Group I). While supplementation with glycyrrhizic acid in Group III and group IV attenuated the immunostaining of connexin-43, Bcl-2 and survivin as compared to Group II. However, there were no significant differences in the immunostaining of connexin-43, Bcl-2 and survivin 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. 11, 13, 14)

4. Discussion

In our previous study, we have shown that glycyrrhizic acid is effective in suppressing the DMH-induced early markers of colon cancer i.e., aberrant crypt foci (ACF) and mucin depleted foci (MDF) plausibly via regulating the hyperproliferation, inflammatory markers, angiogenic switch and apoptotic cascades. But earlier it has been reported that the natural compound i.e., genistein suppresses the development of ACF but enhance experimental colon cancer (Rao et al. 1997). Therefore the present study was designed to evaluate the chemopreventive efficacy of glycyrrhizic acid against DMH-induced colon tumorigenesis in Wistar rats and further to assess
its role in regulating the hyperproliferation, inflammation, angiogenesis and apoptosis. Glycyrrhizic acid treatment reduced the tumor incidence and tumor multiplicity in group III (I group) as compared to group II (DMH treated group) but not at the significant level while glycyrrhizic acid treatment significantly (p<0.05) reduced the tumor incidence in group IV (PI group) as compared to group II (DMH treated group). Most of the tumors were present in the distal colon and it has been reported that the propensity of the distal colon for the development of DMH-induced colonic tumors was higher as compared to proximal colon (Barth et al. 2005). These findings suggest that glycyrrhizic acid suppresses the development of DMH induced colon tumorigenesis in Wistar rats.

Histological findings revealed that control group showed normal histoarchitecture with mild infiltration of inflammatory cells as well as normal mucosal glandular structure while DMH-treated group exhibited (Group II) exhibited in situ adenocarcinoma with papillary pattern, dysplastic zone, marked cellular atypia, loss of goblet cell differentiation, nuclear pseudostratification, high nucleus to cytoplasmic ratio and massive inflammatory cells infiltration. In group III and IV, glycyrrhizic acid supplementation reduces the severity of DMH induced colonic tumors via reducing the infiltration of inflammatory cells within the colonic tumors.

The normal human colonic mucosa and the distal part of the rat colon predominantly secrete sulphomucin (brown color). In the earlier studies, it has been shown that sialomucins (blue color), instead of sulphomucins, are predominantly present in the colonic mucosa from patients with colon cancer and dysplastic foci observed in the distal colon of carcinogen-treated rats (Filipe and Branfoot 1974; Filipe 1975; Wargovich et al. 1983; Sandforth et al. 1988). The distal
colon shows a similar pattern of mucus production with that of normal human colonic mucosa which predominantly secretes sulphomucin; therefore we studied only the distal part of the rat colon. In the precancerous state, there is a shift from sulphomucin to sialomucin secretion in the human as well as in distal rat colon (Filipe and Branfoot 1974; Filipe 1975). In this study, there is predominance of sialomucin (blue color) within the colonic tumors in DMH treated group as compared to control group which showed the predominance of sulphomucin (brown color) while treatment with glycyrrhizic acid attenuated this shifting from sulphomucin to sialomucin in the post-initiation group.

Mast cells, one of the inflammatory cells, play an important role in angiogenesis and also form an important tumour-promoting component of the cellular stroma which promotes tumor growth (Soucek et al. 2007; Gounaris et al. 2007). Influx of mast cells is the sign of initiation of inflammation. Earlier reports 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). In this study, it was observed that there is intense influx of mast cells in the submucosal layer below the colonic tumors in DMH treated group while there is no mast cells influx in control group. Treatment with glycyrrhizic acid markedly reduced the influx of mast cells within in the sub-mucosal layer in group III (I group) and group IV (PI group) as compared to group II (DMH treated group). No influx of mast cells in group V (only GA treated group).

The transcription factor NF-κB is the key molecular underpinning and the probable connecting link between inflammation and cancer (Karin et al. 2002). In inflammatory cells as well as in cells at risk of transformation by carcinogens, NF-κB mediates the transactivation of genes encoding cyclooxygenase-2 (COX2), inducible nitric oxide synthase (iNOS), angiogenic factors (viz.,
VEGF) and anti-apoptotic factors (viz., BCL-2, Survivin), (Orlowski et al. 2002; Mantovani et al. 2008). NF-kB is constitutively activated in various solid malignancies and it has also been shown that enhanced NF-kB activation was observed in human colon adenoma and cancer tissues (Karin et al. 2002; Lind et al. 2001; Hardwick et al. 2001).

Our findings showed that in DMH-treated group there is more NF-kB immunopositive staining within the colonic tumor as well in adjacent to tumor tissue as compared to control group while treatment with glycyrrhizic acid markedly attenuated the NF-kB immunopositive staining. In DMH-treated group, there is more immunopositive staining of other inflammatory mediators i.e., COX-2 and iNOS within the colonic tumor as well in adjacent to tumor tissue as compared to control group. While treatment with glycyrrhizic acid markedly attenuated the immunopositive staining of COX-2 and iNOS. Our results strongly suggest that glycyrrhizic acid has strong anti-inflammatory potential and these results corroborated with previous reports.

Further, in the present study it was observed that in DMH-treated group there is more VEGF immunopositive staining within the colonic tumors as well in adjacent to tumor tissue 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.

Proliferating cell nuclear antigen (PCNA) is a 36kD protein identified as an auxiliary protein of DNA polymerase δ which is synthesized between the late G1 and S phases and it is a cell proliferation marker (Miyachi et al. 1978; Takasaki et al. 1981). 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., PCNA (Cherng et al. 2011).

Ki-67 is another 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 PCNA and Ki-67 immunopositive staining within the colonic tumor as well as adjacent to tumor tissue as compared to control group while treatment with glycyrrhizic acid attenuated the PCNA and 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.

Connexins make up a gene family encoding proteins that form intercellular channels known as gap junctions. Decreases in connexin expression and loss of intercellular communication have been associated with the malignant phenotype in some animal and human cells (Grossman et al. 1994). Cx43 as well as other connexin isoforms are frequently downregulated in tumors, resulting in loss of gap junctional intercellular communication (Leithe et al. 2006; Mesnil et al. 2005). Connexin-43 acts as a tumor suppressor gene (Zhang et al. 2003; Mograbi et al. 2003) and mutations in connexin-43 are involved in advanced stages of progression of human colon cancer towards malignancy (Dubina 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).
Apoptosis is tightly regulated by apoptosis-promoting factors such as p53 and caspases, and anti-apoptotic factors such as Bcl-2 and Survivin. 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 regulates the expression of Bcl-2 (Miyashita et al. 1994) and induces apoptosis by releasing cytochrome c from the mitochondria and Bcl-2 an anti-apoptotic protein, prevent cytochrome c release presumably by binding and inhibition of Bax and Bak (Ghavami et al. 2009). Bcl-2 is involved in the pathogenesis of hematologic and epithelial malignancies, including carcinomas of the lung, colon, breast, and prostate,(Keshgegian et al. 1998; Sinicrope et al. 1995; Dosaka-Akita et al. 1999; Meterissian et al. 2001; Nakamura et al. 1997).

Survivin is an inhibitor of apoptosis protein (IAP) abundantly expressed during normal development and in neoplastic cells but is rarely present in adult tissues, suggesting that deregulation of survivin expression may be involved in tumorigenesis (Ambrosini et al. 1997; Tamm et al. 1998; Carter et al. 2001).

Bcl-2 and survivin contribute independently to embryonic and fetal development (LeBrun et al. 1993; Adida et al. 1998). As inhibitors of apoptosis, both proteins have been implicated in promoting cancer, although the distribution of Bcl-2 and survivin expression varies widely in both normal and transformed cell types. Survivin can bind specifically to the terminal effector cell death proteases such as caspase-3 and inhibits its activity and consequently inhibits apoptosis (Lu et al. 1996).
In this study, it was observed that DMH-treated group has reduced immunopositive staining of connexin-43 as well as p53 within the colonic tumors as well as in adjacent to tumor tissue as compared to control group while treatment with glycyrrhizic acid significantly attenuated the immunopositive staining of connexin-43 and p53.

The present findings exhibited that, in DMH treated group, there was reduced immunopositive staining of Bcl-2 and survivin within the colonic tumors as well as in adjacent to tumor tissue and supplementation with glycyrrhizic acid normalizes the immunopositive staining of Bcl-2 and survivin.

It was also observed that DMH-treated group has reduced immunopositive staining of cleaved caspase-3 within the colonic tumors as compared to control group while treatment with glycyrrhizic acid significantly enhanced the immunopositive staining of 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).

It can be concluded from the findings of the present study that glycyrrhizic acid has chemopreventive potential against DMH-induced colon tumorigenesis in Wistar rats. The precise mechanism of chemopreventive action of glycyrrhizic acid against DMH induced colon tumorigenesis is still unknown but the plausible mechanism may be through the attenuation of hyperproliferative responses, inflammatory markers, angiogenic markers and apoptotic responses in the colon of Wistar rats. Further studies are warranted to elucidate the exact mechanism of action of glycyrrhizic acid.
Figure 1: Morphological appearance of colonic tumors

Table 1: Incidence of colonic tumors and the number of tumors per tumor-bearing rat

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of rats</th>
<th>No. of Tumor-bearing rats</th>
<th>Tumor incidence* (%)</th>
<th>Total tumor number</th>
<th>No. of tumors/tumor-bearing rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>DMH</td>
<td>8</td>
<td>7^a</td>
<td>87^a</td>
<td>10^b</td>
<td>1.4^a</td>
</tr>
<tr>
<td>DMH+GA (I gp)</td>
<td>8</td>
<td>6</td>
<td>75</td>
<td>8</td>
<td>1.33</td>
</tr>
<tr>
<td>DMH+GA (PI gp)</td>
<td>8</td>
<td>4^b</td>
<td>50^b</td>
<td>6^b</td>
<td>1^b</td>
</tr>
<tr>
<td>GA only</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>nil</td>
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</tr>
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</table>

*(Number of tumor-bearing rats/total number of rats in each group) X 100
Values are expressed as mean ± SD
Values not sharing common superscript (a and b) are significant with each other at P<0.05.
Figure 2. 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 with mild inflammatory cells infiltration while DMH treated group (Group II) exhibited in situ adenocarcinoma with papillary pattern, dysplastic zone, marked cellular atypia, loss of goblet cell differentiation, nuclear pseudostratification, high nucleus to cytoplasmic ratio and massive inflammatory cells infiltration. In group III and IV, glycyrrhizic acid supplementation lessens the severity of DMH induced colonic tumors as compared to DMH treated group. 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).
Figure 3. Effects of glycyrrhizic acid and DMH on the colonic sulphomucin and sialomucin.

Figure 3. 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 within the colonic tumors. While treatment with glycyrrhizic acid attenuated this shifting from sulphomucin to sialomucin in Gp III and Gp IV as compared to DMH treated group. Insets at the right panel show a magnified view (40x magnifications) of the insets showed at the left panel (10x magnifications).
Figure 4. Effects of glycyrrhizic acid and DMH on the colonic mast cells infiltration.

Figure 4. 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 massive infiltration of mast cells in the sub-mucosal layer below the lamina propria of the colonic tumors section. Supplementation 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. Original magnification: 40x
Figure 5. Immunohistochemical staining of NF-kB

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.) (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 within the colonic tumors as well as in adjacent to tumor 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 6. Immunohistochemical staining of COX-2

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.) (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 within the colonic tumors as well as in adjacent to tumor 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 7. Immunohistochemical staining of iNOS

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). 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 within the colonic tumors as well as in adjacent to tumor 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 8. Immunohistochemical staining of VEGF

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.) + 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). 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 within the colonic tumors as well as in adjacent to tumor 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 9. Immunohistochemical staining of Ki-67

Figure 9. 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 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 within the colonic tumors as well as in adjacent to tumor 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 10. Immunohistochemical staining of PCNA

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 PCNA and light blue colour indicates nuclear haematoxylin staining. The colonic section of DMH-treated group (Group II) has more PCNA immunopositive staining as indicated by brown colour within the colonic tumors as well as in adjacent to tumor as compared to control group (Group I). While treatment with glycyrrhizic acid in Group III and IV reduced PCNA immunostaining as compared to Group II. However there was no significant difference in the PCNA immunostaining in Group V as compared to Group I. Original magnification: 40x.
Figure 11. Fluorescent immunohistochemical staining of Connexin-43

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 fluorescent immunohistochemical analyses, green colour that fluoresces indicates specific immunostaining of connexin-43. The colonic section of DMH treated group (Group II) has reduced connexin-43 immunopositive staining (arrows) within the colonic tumors as well as adjacent to tumor as indicated by green 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 12. Immunohistochemical staining of p53

Figure 12. 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 within the colonic tumors as well as adjacent to tumor 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 13. Fluorescent immunohistochemical staining of Survivin

Figure 13. 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 enhanced Survivin immunopositive staining (arrows) within the colonic tumors as well as adjacent to tumor as indicated by green colour as compared to control group (Group I) while treatment with glycyrrhizic acid attenuated Survivin immunostaining in Group III and IV as compared to Group II. However, there was no significant difference in the Survivin immunostaining in Group V as compared to Group I. Original magnification: 40x.
Figure 14. Fluorescent immunohistochemical staining of Bcl-2

Figure 13. 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 Bcl-2 and red colour indicates nuclear propidium iodide staining. The colonic section of DMH treated group (Group II) has enhanced Bcl-2 immunopositive staining (arrows) within the colonic tumors as well as adjacent to tumor as indicated by green colour as compared to control group (Group I) while treatment with glycyrrhizic acid attenuated Bcl-2 immunostaining in Group III and IV as compared to Group II. However, there was no significant difference in the Bcl-2 immunostaining in Group V as compared to Group I. Original magnification: 40x.
Figure 15. Immunohistochemical staining of Cleaved Caspase-3

Figure 12. 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 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 within the colonic tumors as well as adjacent to tumor 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.