Chapter VII

Chemopreventive efficacy of *Fagonia cretica*, *Carum carvi* and Diosmin against Diethylnitrosamine (DEN) initiated and 2-Acetylaminofluorene (2-AAF) promoted Hepatocarcinogenesis in Wistar rats
Chapter VII

Chemoprevention of Hepatocarcinogenesis in Wistar rats...

1. Introduction

Cancer is a broad term used for identifying a large number of diseases. The common feature of these diseases is the ability of uncontrolled cell proliferation that cannot be checked by the normal cell kinetics regulators (Trosko, 2001). A normal cell suddenly turns into a rogue cell and start dividing continuously without check, leading to the development of solid lumps (tumors) or an abnormal rise in the number of dispersed cells (Tsao et al., 2004). Cancer originates in our own cells, but several factors, both intrinsic and external to the body, which influence our daily life, can add to the life time cancer risk. While cancer, as such, is not infectious, some infections can act as a stimulus to induce and promote cancer development (Camargo et al., 1999). In addition, environmental pollutants like many chemicals, industrial effluents, some therapeutic drugs, and mutagenic agents, including ionizing radiation, can increase the incidence of cancer. About 50% of all cancers are attributed to lifestyle, e.g., diet, tobacco habits and alcohol consumption, and exposure to industrial toxins (Pitot HC and Dragan, 1991; Barrett and Anderson, 1993; Farmer, 1994; Weisburger 1999; Minamoto et al., 2000; Lutz 2002).

Hepatocellular carcinoma (HCC) is a primary malignancy of hepatocytes which accounts for 80% of all primary liver cancers and ranks globally as the fourth leading cause of cancer related death (Abrams and Marsh, 2010; Jemal et al., 2011). Chronic infection with hepatitis B and C virus being the major risk factors for hepatocarcinogenesis, several etiological risk factors have also been identified including alcohol abuse, exposure to aflatoxin B1, and nonalcoholic fatty liver change (Kohle et al., 2008). HCC frequently arises in patients with liver cirrhosis, the result of a complex pathogenesis involving chronic hepatitis, necrosis, and regenerative fibrosis. Since terminal stage HCC can rarely be cured by conventional therapeutic approaches, the final remaining therapeutic choices are surgical resection and transplantation (Kaiser et al., 2010). However, the survival rate of HCC patients who have undergone surgical resection is known to be less than 25% (Wild and Hall, 2000). Therefore, development of an effective cancer chemopreventive agent is urgently needed for hepatic cancer prevention. Cancer chemoprevention is defined as an attempt to suppress or prevent the carcinogenic progression with natural or synthetic chemical agents.

Diethylnitrosamine (DEN) induced and 2-acetylaminofluorene (2-AAF) promoted hepatocarcinogenesis model given by Solt and Farber (1976) has been widely used by our laboratory and other investigators to evaluate the cancer chemopreventive efficacy of several compounds (Sehrawat and Sultana, 2006; Sultana et al., 2008). It has been demonstrated that administration of 2-AAF after DEN initiation in Wistar rats exerts promotional effect by stimulating growth of focal cells rather than by suppression of proliferation of normal cells.
(Gupta et al., 1988). The positive foci development and cellular proliferation have been directly related to dietary concentration of 2-AAF (Tiwawech et al., 1997). 2-Nitrosoflourine, a metabolite of 2-AAF was shown to induce redox cycling leading to superoxide production capable of reacting to DNA (Klohn et al., 1995).

Recent studies have shown that reactive oxygen species play an important role in tumor development (Kumar et al., 2008; Ishikawa et al., 2008). ROS can be produced from endogenous sources, such as from mitochondria, peroxisomes, and inflammatory cell activation (Klaunig and Kamendulis, 2004); and exogenous sources, including environmental agents, pharmaceuticals, and industrial chemicals. These ROS then, in turn, may cause DNA, protein, and/or lipid damage, leading to changes in chromosome instability, genetic mutation, and/or modulation of cell growth that may result in cancer. Oxidative stress is believed to be the main mechanism behind the DEN initiated and 2-AAF promoted hepatocarcinogenesis. Increased cellular oxidants within the cell leads to the induction of lipid peroxidation, xanthine oxidase activity, glutathione depletion, decrease in antioxidant (catalase, glutathione reductase and glutathione peroxidase) and phase-II detoxifying (glutathione-S-transferase and quinone reductase) enzyme activities. These cellular oxidants (free radicals) and altered antioxidants (antioxidant enzymes) further leads to the activation of tumor necrosis factor (TNF-α) and various transcription factors such as nuclear factor erythroid 2-related factor 2 (NF-E2/rf2 or Nrf2) (Kensler et al., 2007), mitogen-activated protein (MAP) kinase (Benhar et al., 2002; Zhang and Liu, 2002), and NF-κB pathways (Pantano et al., 2006). Moreover, mutation studies have also suggested that chronic oxidative stress, particularly from chronic inflammation, is associated with carcinogenesis (Hwang and Bowen 2007). It has been demonstrated that during carcinogenesis process there is elevation in the expression of inflammatory markers, especially COX-2 and inducible NOS (Subbaramaiah et al., 1996; Kelley et al., 1997).

Carcinogenesis, a multistage process most often described as uncontrolled proliferation of cells. Their growth is associated with the action of certain proliferative proteins such as proliferating cell nuclear antigen (PCNA) and Ki-67. PCNA, a non-histamine nuclear protein, and is a specific marker of cell division. Its action is associated with DNA polymerase, synthesized shortly before the S-phase of the cell cycle (Kelman, 1997). PCNA expression has been found to correlate with the degree of malignancy, vascular infiltration, distant metastasis and survival. This antigen has been described as one of the biomarker of hepatocellular carcinoma (Oyama et al., 2002). Ki-67, an excellent marker of mitotic index and the fraction of dividing cells, is detected during all phases of the cell cycle (G1, S, G2 and mitosis), except for the G0-phase (Sahin et al., 1994). Expression of Ki-67 like PCNA also correlates with the course of neoplastic disease and can
thus be used to assess patient survival and progression of cancer. The level of Ki-67 expression is of great prognostic significance in carcinoma of the prostate, breast, liver, in malignant melanoma, malignant lymphoma and lung cancer (Scholzen and Gerdes, 2000; Cambruzzi, et al., 2005).

Chemoprevention, a novel and appealing strategy, deals with the inhibition, reversal or suppression of carcinogenesis by the use of natural or synthetic agents (F'guyer et al., 2003). The possible mechanism so far reported for the chemopreventive potential of natural products include carcinogen detoxification, suppression of genetic mutation, suppression of cell proliferation, induction of apoptosis and modulation of the immune system (Dorai and Aggarwal, 2004). Agents that possess anti-mutagenic and antioxidant potential have the ability to exert striking inhibitory effects on diverse cellular events associated with multistage carcinogenesis (Crowell, 2005). Fagonia cretica is astringent, febrifuge and prophylactic against small-pox. The plant is bitter and used for the treatment of fever, thirst, vomiting, dysentery, asthma, urinary discharges, liver trouble, typhoid, toothache, stomach troubles and skin diseases (Baquar, 1989; Razi et al., 2011; Rawal et al., 2004). It has also been used as a remedy for cancer in the indigenous system of medicine (Saeed, 1969). Carum carvi, one of the main and most widely used wild spice plants in many countries and possess many medicinal properties including, antidispeptic (Holtmann 2003), antispasmodic (Eddouks, 2004), antiulcerogenic (Khayyal, 2001), antibacterial (Singh, 2002), antitumor (Kamaleeswari, 2006), antiproliferative (Nakano, 1998), antioxidant (Kamaleeswari, 2006), antihyperglycemic (Eddouks, 2004), antihyperlipidaemic (Lemhadri, 2006) and diuretic (Lahlou, 2007) activities. Diosmin, a bioflavonoid (3',5,7-trihydroxy-4'-methoxyflavone 7-rutinoside) present in citrus fruits (Nogata et al., 1994) exhibit tremendous biological activities including antioxidant property, anti-inflammatory effect, and inhibition of prostaglandin synthesis (Dung et al., 2012; Silambarasan and Raja, 2012; Srinivasan and Pari, 2012).

The aim of the present study was to access the chemopreventive efficacy of Fagonia cretica, Carum carvi and diosmin against DEN initiated and 2-AAF promoted hepatocarcinogenesis in Wistar rats in correlation with the alterations in the expressions of necrosis factors (TNF-α), transcription factors (NF-κB, MAP kinase), proliferation markers (PCNA, Ki67), inflammatory cytokines (COX-2, iNOS) and histopathology.
2. Treatment regimen

DEN initiated and 2-AAF promoted hepatocarcinogenesis study (Solt and Farber Method)

In this study, chemopreventive efficacy of modulators (*Fagonia cretica, Carum carvi* and *diosmin*) on DEN initiated and 2-AAF promoted hepatocarcinogenesis were evaluated; the rats were divided into four groups of 12 rats per group. The complete treatment regimen followed in tumor study is illustrated in figure below and the detail is also given:

- **Group I** animals received only normal saline (0.9%) by oral gavage once daily for 6 weeks and served as controls.

- **Group II** has been administered with an i.p injection of DEN (200mg/kg body weight). In addition, animals of the group II has been given 2-AAF (0.02% w/w) in diet after 14 days of initiation with DEN for six weeks. Moreover, all animals were subjected to partial hepatectomy (PH) after one week of 2-AAF dietary administration (i.e., on 21st day)

- **Group III** was given same treatment as group II and were also pretreated (1 week before DEN administration) by modulator (*Fagonia cretica, Carum carvi, diosmin*) once daily by oral gavage, at a dose D1 for a period of 9 weeks.

- **Group IV** was given same treatment as group II and were also pretreated (1 week before DEN administration) by modulator (*Fagonia cretica, Carum carvi, diosmin*) once daily by oral gavage, at a dose D2 for a period of 9 weeks.

At the end of 22 weeks, all the animals were sacrificed under light ether anesthesia. Their livers were quickly removed and processed for various molecular, histopathological and immune-histochemical studies.
Experimental design of DEN initiated and 2-AAF promoted hepatocarcinogenesis in Wistar rats by Solt and Farber method
3. Results

3.1. Study 1: Chemoprevention of DEN initiated and 2-AAF promoted \textit{Hepatocarcinogenesis} by \textit{Fagonia cretica} in Wistar rats

3.1.1. Effect of \textit{Fagonia cretica} on the development of tumor nodules during DEN initiated and 2-AAF promoted \textit{hepatocarcinogenesis}

Livers from the rats of the control group showed no tumors. It has been observed that there is 90% tumor incidence in rats of group II (DEN + 2-AAF + PH). While as administration of \textit{Fagonia cretica} at doses D1 (100mg/kg b wt) and D2 (200mg/kg b wt) showed 58% and 25% of tumor incidences. Reduction in the tumor incidence confirmed the chemopreventive efficacy of \textit{Fagonia cretica} (Table 1).

3.1.2. Effect of \textit{Fagonia cretica} on expression of PCNA, Ki67, COX-2, iNOS, P38, P53 and NFκB in hepatic tissue during DEN initiated and 2-AAF promoted \textit{hepatocarcinogenesis}

Hepatic expressions of PCNA, Ki67, COX-2, iNOS, P38, P53 and NFκB have been shown in the figures 2(I), 2(II), 3(I), 3(II), 4(I), 4(II) and 5 respectively. Brown colour clearly indicates the more number of cells having expressions of these proteins in the group II as compared to that of group I. Treatment with \textit{Fagonia cretica} in the group III reduced the number of cells showing expression of these proteins. For immunohistochemical analysis (Original magnification: x400).

3.1.3. Effect of \textit{Fagonia cretica} on hepatic histopathology during DEN initiated and 2-AAF promoted \textit{hepatocarcinogenesis}

Histopathological findings on liver sections from various experimental groups of animals are illustrated in Figure 6. The hepatic sections from normal animals (Group I) revealed normal liver parenchyma with the typical architecture characterized by granulated cytoplasm, central vein and small uniform nuclei (Fig. 6A). Rats of group II showed abnormal architecture with the presence of irregular-shaped cytoplasm and enlarged nuclei (Fig. 6B). A large number of abnormal hepatocytes were observed which were binucleated and extensive vacuolation was noticed in the cytoplasm surrounding the nucleus with masses of eosinophilic material. Treatment of rats with \textit{Fagonia cretica} at a dose of 100 mg/kg body weight (Group III) improved the hepatocellular architecture with more regular and less altered hepatocytes when compared to Group II (Fig. 6C). The cellular architecture of liver sections from rats that received \textit{Fagonia cretica} at 200 mg/kg (Group D) was comparable with that of the normal animals. The hepatocytes from this group had a compact cytoplasm (Fig. 6D) which indicates the absence of hepatotoxicity.
Table 1: Summary of tumor data showing effects of *Fagonia cretica* treatment on DEN initiated and 2-AAF promoted hepatocarcinogenesis in Wistar rats.

<table>
<thead>
<tr>
<th></th>
<th>No of Rats/group</th>
<th>Animal Survived</th>
<th>Rats with visible tumors</th>
<th>Incidence of Rat tumors (%)</th>
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<td><strong>Control</strong></td>
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<td>0</td>
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<td><strong>DEN+2-AAF+PH</strong></td>
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<td>90</td>
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<tr>
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Figure 1: Morphological examination of rat liver tissue at the end of the study

Macroscopically visible hepatic nodules are shown by arrows. Representative livers were excised from several groups: (A) normal (Group I) showing absence of nodules; (B) DEN + 2-AAF + PH group (Group II) showing a large nodule; (C) *Fagonia cretica* (100 mg/kg body weight) + DEN + 2-AAF + PH group (Group III), showing small nodules; (D) *Fagonia cretica* (200 mg/kg body weight) + DEN + 2-AAF group (Group IV) with no visible nodules.
Figure 2: Expression of Proliferation markers (PCNA and Ki67) in liver tissue during DEN initiated and 2-AAF promoted hepatocarcinogenesis

Photomicrograph represents the effect of Fagonia cretica treatment on PCNA (I) and Ki67 (II) expressions. Brown color indicates specific immunostaining of PCNA and Ki67, and light color indicates haematoxylin staining. (A) Control group (Group I) showing no expression; (B) DEN + 2-AAF+ PH group (Group II) showing high expression of PCNA and Ki67; (C) Fagonia cretica (100 mg/kg body weight) + DEN + 2-AAF + PH group (Group III) showing less expression of
PCNA and Ki67; (D) *Fagonia cretica* (200 mg/kg body weight) + DEN + 2-AAF group (Group IV) showing no expression of PCNA and Ki67 (Original magnification: x400).

**Figure 3:** Expression of inflammatory markers (COX-2 and iNOS) in liver tissue during DEN initiated and 2-AAF promoted hepatocarcinogenesis

Photomicrograph represents the effect of *Fagonia cretica* treatment on COX-2 (I) expressions. Brown color indicates specific immunostaining of COX-2, and light color indicates haematoxylin staining. (A) Control group (Group I) showing no expression; (B) DEN + 2-AAF + PH group (Group II) showing high expression of COX-2; (C) *Fagonia cretica* (100 mg/kg body weight) + DEN + 2-AAF + PH group (Group III).showing less expression of COX-2 (D) *Fagonia cretica* (200 mg/kg body weight) + DEN + 2-AAF group (Group IV) showing no expression of COX-2 (Original magnification: x400).
Figure 3 (II): Effect of *Fagonia cretica* on iNOS expression in rat liver tissue

Photomicrograph represents the effect of *Fagonia cretica* treatment on iNOS (II) expressions. Brown color indicates specific immunostaining of iNOS, and light color indicates haematoxylin staining. (A) Control group (Group I) showing no expression; (B) DEN + 2-AAF + PH group (Group II) showing high expression of iNOS; (C) *Fagonia cretica* (100 mg/kg body weight) + DEN + 2-AAF + PH group (Group III) showing less expression of iNOS; (D) *Fagonia cretica* (200 mg/kg body weight) + DEN + 2-AAF group (Group IV) showing no expression of iNOS (Original magnification: x400).
Figure 4: Expression of P38 (I) and P53 (II) in liver tissue during DEN initiated and 2-AAF promoted hepatocarcinogenesis

Photomicrograph represents the effect of *Fagonia cretica* treatment on P38 (I) and P53 (II) expressions. Brown color indicates specific immunostaining of COX-2 and iNOS, and light color indicates haematoxylin staining. (A) Control group (Group I); (B) DEN + 2-AAF + PH group (Group II); (C) *Fagonia cretica* (100 mg/kg body weight) + DEN + 2-AAF + PH group (Group III); (D) *Fagonia cretica* (200 mg/kg body weight) + DEN + 2-AAF group (Group IV) (Original magnification: x400).
Figure 5: Effect of *Fagonia cretica* on the expression of NFκB in liver tissue during DEN initiated and 2-AAF promoted hepatocarcinogenesis

![Photomicrograph](image)

NFκB

Photomicrograph represents the effect of *Fagonia cretica* treatment on NFκB expression. Brown color indicates specific immunostaining of NFκB. (A) Control group (Group I) showing no expression of NFκB; (B) DEN + 2-AAF + PH group (Group II) showing high expression of NFκB; (C) *Fagonia cretica* (100 mg/kg body weight) + DEN + 2-AAF + PH group (Group III) showing less expression of NFκB; (D) *Fagonia cretica* (200 mg/kg body weight) + DEN + 2-AAF group (Group IV) showing no expression of NFκB (Original magnification: x400).
Figure 6: Effect of *Fagonia cretica* on hepatic histopathology during DEN initiated and 2-AAF promoted hepatocarcinogenesis

Figure 6: Histopathological profiles of representative liver tissues from various experimental animals. (A) Normal untreated rat liver (Group I) showing normal cellular architecture (H&E; original magnification ×400). (B) DEN + 2-AAF + PH group (Group II) showing areas of aberrant hepatocellular phenotype with variation in nuclear size, and proliferating cells (H&E; original magnification ×400). (C) liver section from *Fagonia cretica* (100 mg/kg body weight) + DEN + 2-AAF + PH group (Group III) showing moderate improvement of hepatic histopathology over group B (H&E; original magnification ×400). (D) Section from *Fagonia cretica* (200 mg/kg body weight) + DEN + 2-AAF + PH group (Group IV) showing hepatocytes maintaining near-normal architecture (H&E; original magnification ×400).
3.2. Study 2: Chemopreventive efficacy of *Carum carvi* against DEN initiated and 2-AAF promoted hepatocarcinogenesis in Wistar rats

3.2.1. Effect of *Carum carvi* on the development of tumor nodules during DEN initiated and 2-AAF promoted hepatocarcinogenesis

Livers from the rats of the control group showed no tumors. It has been observed that there is 90% tumor incidence in rats of group II (DEN + 2-AAF + PH). While as administration of *Carum carvi* at doses D1 (100mg/kg b wt) and D2 (200mg/kg b wt) showed 66% and 41% of tumor incidences. Reduction in the tumor incidence confirmed the chemopreventive efficacy of *Carum carvi* (Table 2; Figure 7).

3.2.2. Effect of *Carum carvi* on the expression of PCNA, Ki67, COX-2, iNOS, P38, P53 and NFκB in hepatic tissue during DEN initiated and 2-AAF promoted hepatocarcinogenesis

Hepatic expressions of PCNA, Ki67, COX-2, iNOS, P38, P53 and NFκB have been shown in the figures 8(I), 8(II), 9(I), 9(II), 10(I), 10(II) and 11 respectively. Brown colour clearly indicates the more number of cells having expressions of these proteins in the group II as compared to that of group I. Treatment with *Fagonia cretica* in the group III reduced the number of cells showing expression of these proteins. For immunohistochemical analysis (Original magnification: x400).

3.2.3. Effect of *Carum carvi* on hepatic histopathology during DEN initiated and 2-AAF promoted hepatocarcinogenesis

Histopathological findings on liver sections from various experimental groups of animals are illustrated in Figure 12. The hepatic sections from normal animals (Group I) revealed normal liver parenchyma with the typical architecture characterized by granulated cytoplasm, central vein and small uniform nuclei (Fig. 12A). Rats of group II showed abnormal architecture with the presence of irregular-shaped cytoplasm and enlarged nuclei (Fig. 12B). A large number of abnormal hepatocytes were observed which were binucleated and extensive vacuolation was noticed in the cytoplasm surrounding the nucleus with masses of eosinophilic material. Treatment of rats with *Carum carvi* at a dose of 100 mg/kg body weight (Group III) improved the hepatocellular architecture with more regular and less altered hepatocytes when compared to Group II (Fig. 12C). The cellular architecture of liver sections from rats that received *Carum carvi* at 200 mg/kg (Group D) was comparable with that of the normal animals. The hepatocytes from this group had a compact cytoplasm (Fig. 12D) which indicates the absence of hepatotoxicity.
Table 2: Summary of tumor data showing effects of *Carum carvi* treatment on DEN initiated and 2-AAF promoted hepatocarcinogenesis in Wistar rats.

<table>
<thead>
<tr>
<th></th>
<th>No of Rats/group</th>
<th>Animal Survived</th>
<th>Rats with visible tumors</th>
<th>Incidence of Rat tumors (%)</th>
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<td>Control</td>
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<td>12</td>
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<tr>
<td>DEN+2-AAF+ PH</td>
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<tr>
<td>DEN+2-AAF+ PH + <em>Carum carvi</em> (200mg/kg b. wt)</td>
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Figure 7: Morphological examination of rat liver tissue at the end of the study

Macroscopically visible hepatic nodules are shown by arrows. Representative livers were excised from several groups: (A) normal (Group I) showing absence of nodules; (B) DEN + 2-AAF + PH group (Group II) showing a large nodule; (C) Carum carvi (100 mg/kg body weight) + DEN + 2-AAF + PH group (Group III) showing small nodule; (D) Carum carvi (200 mg/kg body weight) + DEN + 2-AAF group (Group IV) with no visible nodules.
Figure 8: Effect of *Carum carvi* on the expression of Proliferation markers (PCNA and Ki67) in liver tissue during DEN initiated and 2-AAF promoted hepatocarcinogenesis

Photomicrograph represents the effect of *Fagonia cretica* treatment on PCNA (I) and Ki67 (II) expressions. Brown color indicates specific immunostaining of PCNA and Ki67, and light color indicates haematoxylin staining. (A) Control group (Group I) showing no expression; (B) DEN + 2-AAF+ PH group (Group II) showing high expression of PCNA and Ki67; (C) *Carum carvi* (100 mg/kg body weight) + DEN + 2-AAF + PH group (Group III), showing less expression of PCNA and Ki67; (D) *Carum carvi* (200 mg/kg body weight) + DEN + 2-AAF group (Group IV), showing no expression of PCNA and Ki67 (Original magnification: x400).
Figure 9: Effect of *Carum carvi* on the expression of inflammatory markers (COX-2 and iNOS) in liver tissue during DEN initiated and 2-AAF promoted hepatocarcinogenesis

Photomicrograph represents the effect of *Carum carvi* treatment on COX-2 (I) and iNOS (II) expressions. Brown color indicates specific immunostaining of COX-2 and iNOS, and light color indicates haematoxylin staining. (A) Control group (Group I) showing no expression; (B) DEN + 2-AAF+ PH group (Group II) showing high expression of COX-2 and iNOS; (C) *Carum carvi* (100 mg/kg body weight) + DEN + 2-AAF + PH group (Group III).showing less expression of COX-2 and iNOS; (D) *Carum carvi* (200 mg/kg body weight) + DEN + 2-AAF group (Group IV) showing no expression of COX-2 and iNOS (Original magnification: x400)
Figure 10: Effect of treatment of *Carum carvi* on the expression of P38 (I) and P53 (II) in liver tissue during DEN initiated and 2-AAF promoted hepatocarcinogenesis

Photomicrograph represents the effect of *Carum carvi* treatment on P38 (I) and P53 (II) expressions. Brown color indicates specific immunostaining of COX-2 and iNOS, and light color indicates haematoxylin staining. (A) Control group (Group I); (B) DEN + 2-AAF + PH group (Group II); (C) *Carum carvi* (100 mg/kg body weight) + DEN + 2-AAF + PH group (Group III); (D) *Carum carvi* (200 mg/kg body weight) + DEN + 2-AAF group (Group IV) (Original magnification: x400).
Figure 11: Effect of *Carum carvi* on the expression of NFκB in liver tissue during DEN initiated and 2-AAF promoted hepatocarcinogenesis

Photomicrograph represents the effect of *Carum carvi* treatment on NFκB expression. Brown color indicates specific immunostaining of NFκB. (A) Control group (Group I) showing no expression of NFκB; (B) DEN + 2-AAF+ PH group (Group II) showing high expression of NFκB; (C) *Carum carvi* (100 mg/kg body weight) + DEN + 2-AAF + PH group (Group III) showing less expression of NFκB; (D) *Carum carvi* (200 mg/kg body weight) + DEN + 2-AAF group (Group IV) showing no expression of NFκB (Original magnification: x400).
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Figure 12: Effect of *Carum carvi* on hepatic histopathology during DEN initiated and 2-AAF promoted hepatocarcinogenesis

![Figure 12: Effect of *Carum carvi* on hepatic histopathology during DEN initiated and 2-AAF promoted hepatocarcinogenesis](image)

Figure 6: Histopathological profiles of representative liver tissues from various experimental animals. (A) Normal untreated rat liver (Group I) showing normal cellular architecture (H&E; original magnification ×400). (B) DEN + 2-AAF + PH group (Group II) showing areas of aberrant hepatocellular phenotype with variation in nuclear size, and proliferating cells (H&E; original magnification ×400). (C) Liver section from *Carum carvi* (100 mg/kg body weight) + DEN + 2-AAF + PH group (Group III) showing moderate improvement of hepatic histopathology over group B (H&E; original magnification ×400). (D) Section from *Carum carvi* (200 mg/kg body weight) + DEN + 2-AAF + PH group (Group IV) showing hepatocytes maintaining near-normal architecture (H&E; original magnification ×400).
3.3. **Study 3: Chemopreventive efficacy of Diosmin against DEN initiated and 2-AAF promoted hepatocarcinogenesis in Wistar rats**

3.3.1. **Effect of Diosmin on the development of tumor nodules during DEN initiated and 2-AAF promoted hepatocarcinogenesis**

Livers from the rats of the control group showed no tumors. It has been observed that there is 90% tumor incidence in rats of group II (DEN + 2-AAF + PH). While as administration of Diosmin at doses D1 (10mg/kg b wt) and D2 (20mg/kg b wt) showed 50% and 25% of tumor incidences. Reduction in the tumor incidence confirmed the chemopreventive efficacy of Diosmin (Table 3).

3.3.2. **Effect of Diosmin on the expression of PCNA, Ki67, COX-2, iNOS, P38, P53 and NFκB in hepatic tissue during DEN initiated and 2-AAF promoted hepatocarcinogenesis**

Hepatic expressions of PCNA, Ki67, COX-2, iNOS, P38, P53 and NFκB have been shown in the figures 14(I), 14(II), 15(I), 15(II), 16(I), 16(II) and 17 respectively. Brown colour clearly indicates the more number of cells having expressions of these proteins in the group II as compared to that of group I. Treatment with *Fagonia cretica* in the group III reduced the number of cells showing expression of these proteins. For immunohistochemical analysis (Original magnification: x400).

3.3.3. **Effect of Diosmin on hepatic histopathology during DEN initiated and 2-AAF promoted hepatocarcinogenesis**

Histopathological findings on liver sections from various experimental groups of animals are illustrated in Figure 18. The hepatic sections from normal animals (Group I) revealed normal liver parenchyma with the typical architecture characterized by granulated cytoplasm, central vein and small uniform nuclei (Fig. 18A). Rats of group II showed abnormal architecture with the presence of irregular-shaped cytoplasm and enlarged nuclei (Fig. 18B). A large number of abnormal hepatocytes were observed which were binucleated and extensive vacuolation was noticed in the cytoplasm surrounding the nucleus with masses of eosinophilic material. Treatment of rats with Diosmin at a dose of 10 mg/kg body weight (Group III) improved the hepatocellular architecture with more regular and less altered hepatocytes when compared to Group II (Fig. 18C). The cellular architecture of liver sections from rats that received Diosmin at 20 mg/kg (Group D) was comparable with that of the normal animals. The hepatocytes from this group had a compact cytoplasm (Fig. 18D) which indicates the absence of hepatotoxicity.
Table 3: Summary of tumor data showing effects of Diosmin treatment on DEN initiated and 2-AAF promoted hepatocarcinogenesis in Wistar rats.

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</table>
Figure 13: Morphological examination of rat liver tissue at the end of the study

Macroscopically visible hepatic nodules are shown by arrows. Representative livers were excised from several groups: (A) normal (Group I) showing absence of nodules; (B) DEN + 2-AAF + PH group (Group II) showing a large nodule; (C) Diosmin (10 mg/kg body weight) + DEN + 2-AAF + PH group (Group III), showing small nodule; (D) Diosmin (20 mg/kg body weight) + DEN + 2-AAF group (Group IV) with no visible nodules.
Figure 14: Effect of Diosmin on the expression of Proliferation markers (PCNA and Ki67) in liver tissue during DEN initiated and 2-AAF promoted hepatocarcinogenesis

Photomicrograph represents the effect of Diosmin treatment on PCNA (I) and Ki67 (II) expressions. Brown color indicates specific immunostaining of PCNA and Ki67, and light color indicates haematoxylin staining. (A) Control group (Group I) showing no expression; (B) DEN + 2-AAF+ PH group (Group II) showing high expression of PCNA and Ki67; (C) Diosmin (10 mg/kg body weight) + DEN + 2-AAF + PH group (Group III).showing less expression of PCNA and Ki67; (D) Diosmin (20 mg/kg body weight) + DEN + 2-AAF group (Group IV) showing no expression of PCNA and Ki67 (Original magnification: x400).
Figure 15: Effect of Diosmin on the expression of inflammatory markers (COX-2 and iNOS) in liver tissue during DEN initiated and 2-AAF promoted hepatocarcinogenesis

Photomicrograph represents the effect of Diosmin treatment on COX-2 (I) and iNOS (II) expressions. Brown color indicates specific immunostaining of COX-2 and iNOS, and light color indicates haematoxylin staining. (A) Control group (Group I) showing no expression; (B) DEN + 2-AAF+ PH group (Group II) showing high expression of COX-2 and iNOS; (C) Diosmin (10 mg/kg body weight) + DEN + 2-AAF + PH group (Group III), showing less expression of COX-2 and iNOS; (D) Diosmin (20 mg/kg body weight) + DEN + 2-AAF group (Group IV) showing no expression of COX-2 and iNOS (Original magnification: x400).
Figure 16: Effect of treatment of Diosmin on the expression of P38 (I) and P53 (II) in liver tissue during DEN initiated and 2-AAF promoted hepatocarcinogenesis

Photomicrograph represents the effect of Diosmin treatment on P38 (I) and P53 (II) expressions. Brown color indicates specific immunostaining of COX-2 and iNOS, and light color indicates haematoxylin staining. (A) Control group (Group I); (B) DEN + 2-AAF + PH group (Group II); (C) Diosmin (10 mg/kg body weight) + DEN + 2-AAF + PH group (Group III); (D) Diosmin (20 mg/kg body weight) + DEN + 2-AAF group (Group IV) (Original magnification: x400)
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Figure 17: Effect of Diosmin on the expression of NFκB in liver tissue during DEN initiated and 2-AAF promoted hepatocarcinogenesis

Photomicrograph represents the effect of Diosmin treatment on NFκB expression. Brown color indicates specific immunostaining of NFκB. (A) Control group (Group I) showing no expression of NFκB; (B) DEN + 2-AAF+ PH group (Group II) showing high expression of NFκB; (C) Diosmin (10 mg/kg body weight) + DEN + 2-AAF + PH group (Group III), showing less expression of NFκB; (D) Diosmin (20 mg/kg body weight) + DEN + 2-AAF group (Group IV) showing no expression of NFκB (Original magnification: x400).
Figure 18: Effect of Diosmin on hepatic histopathology during DEN initiated and 2-AAF promoted hepatocarcinogenesis

Figure 6: Histopathological profiles of representative liver tissues from various experimental animals. (A) Normal untreated rat liver (Group I) showing normal cellular architecture (H&E; original magnification ×400). (B) DEN + 2-AAF + PH group (Group II) showing areas of aberrant hepatocellular phenotype with variation in nuclear size, and proliferating cells (H&E; original magnification ×400). (C) Liver section from Diosmin (10 mg/kg body weight) + DEN + 2-AAF + PH group (Group III) showing moderate improvement of hepatic histopathology over group B (H&E; original magnification ×400). (D) Section from Diosmin (20 mg/kg body weight) + DEN + 2-AAF + PH group (Group IV) showing hepatocytes maintaining near-normal architecture (H&E; original magnification ×400).
4. Discussion

Hepatocarcinogenesis initiated by diethylnitrosamine (DEN) followed by 2-acetylaminofluorene and partial hepatectomy (PH) in rodents has been considered as one of the most characterised experimental models of liver cancer, allowing the screening of potential anticancer compounds on various phases of neoplastic transformation and development (Sehrawat and Sultana, 2006; Taha et al., 2010; Khan and Sultana, 2011). Partial hepatectomy (PH) corresponds to the method of surgically inducing hyperplasia for speedy onset of carcinogenic process and enhanced hepatocellular carcinoma development (Best and Coleman, 2007; Park and Suh, 1999). DEN induced preneoplastic foci and neoplastic nodule formation in rodents closely mimics hepatocellular carcinoma development in humans (Peto et al., 1991; Verna et al., 1996; Li et al., 2005). Recently, a cross-species comparison of gene expression patterns has established that DEN induced liver tumors in rodents closely resemble a subclass of human hepatocellular carcinogenesis (Lee et al., 2004), which allows extrapolating the potential chemopreventive effects of a candidate agent in clinical setting.

In the present study, we have investigated the preventive effect of diosmin and methanolic extracts of *Fagonia cretica* and *Carum carvi* on the appearance of hepatic preneoplastic events, utilizing a two stage model of hepatocarcinogenesis initiated with DEN and promoted by 2-AAF. The results of our study clearly indicate a beneficial effect of these modulators on chemically induced rat liver tumorigenesis. Under our experimental conditions, treatment with diosmin, *Fagonia cretica* and *Carum carvi* treatment to DEN and 2-AAF exposed rats resulted in fewer animals developing visible hepatocyte nodules compared to the rats of group II.

Moreover, a large body of experience in both experimental and human disease provides a strong correlation between the number of nodular hyperplasia and hepatocarcinogenesis (Farber and Cameron, 1980; Farber, 1990). In the background of these studies, suppression of nodule growth in number and size of nodules by diosmin, *Fagonia cretica* and *Carum carvi* treatments observed in the present study could be viewed as vital steps for cancer chemoprevention. These findings are of critical importance when considering the fact that persistent nodules are easily recognizable.

Cell proliferation plays a primary role in multistage carcinogenesis and is also believed to be involved in the pathogenesis of hepatocellular carcinogenesis (Farber, 1991; Hirahu et al., 2001). Additionally, the rate of cell proliferation is considered to be a well-established marker for cancer development (Cohen and Ellwin, 1990). Hence, the discovery of agents that can affect abnormal proliferation of liver cells is of immense importance in chemoprevention of hepatocellular carcinogenesis. In the present approach of chemoprevention, we have evaluated the expression of two important proliferation markers viz, PCNA and Ki67 in DEN initiated and 2-AAF
promoted model of hepatocarcinogenesis. The expression of PCNA, have been linked to the late G1 as well as early S-phase of the cell cycle (Lu et al., 2002) and Ki67 expressions in all phases of cell cycles except G0 phase (Sahin et al., 1994). One common approach to study the proliferative status of transformed cells is by detection of PCNA and Ki67 through immuno-histochemical techniques (Eldrige et al., 1993; Itaya et al., 2005). In conjunction with histopathological characteristics, over expression of PCNA and Ki67 are the reliable markers for evaluating tumor differentiation and tumor progression as well as early detection and prognosis of hepatocellular carcinoma (Ng et al., 1994). To investigate the mechanism by which the modulators of the present study (Fagonia cretica, Carum carvi and diosmin) provide chemoprevention of hepatocarcinogenesis, we have examined the extent of cell proliferation in hepatic tissue during DEN initiated and 2-AAF promoted tumorigenesis in the presence and absence of Fagonia cretica/ Carum carvi/diosmin. Our immunohistochemical analysis displays intense immunostaining of PCNA and Ki67 in the preneoplastic liver tissue from animals of group II (DEN + 2-AAF + PH) when compared to controls. The expressions of PCNA and Ki67 in areas of high proliferative activity, as observed in present study, represents an early event in the pathogenesis of hepatic neoplasia and confirms the observations from other reports (Chakraborty et al., 2006; Chodon et al., 2007; Kim et al., 2007; Youssef et al., 2012). Treatment with Fagonia cretica/ Carum carvi/diosmin remarkably decreased the number of PCNA positive cells, indicating its ability to suppress abnormal proliferation of initiated hepatocytes through an antiproliferative activity.

Although diverse mechanisms have been put forward to account for the chemopreventive potential of natural products (Surh, 2003; Lee et al., 2007), attention has recently been focused on signalling molecules mediating in inflammation and cancer. Because of a causal link between inflammation and cancer (Clevers, 2004), targeted blockade of intracellular signalling pathways mediating inflammatory response is now considered a road map for developing molecular target based chemopreventive agents (Surh et al., 2005). Therefore modulation of cellular signalling network involved in induction and activity of COX-2 and/or iNOS has been considered a new paradigm for preventing carcinogenesis (Chung et al., 2007). In the present study, we have found that Fagonia cretica/Carum carvi/diosmin inhibits COX-2 and iNOS expression in rat liver through down regulation of Cox-2 and iNOS in a DEN and 2-AAF induced carcinogenesis model of liver cancer.

NF-κB plays a central role in general inflammation as well as tumorigenesis (Surh et al., 2001). Rapid phosphorylation of IκBα (inhibitory subunit of NF-κB) and its subsequent degradation following exposure of cells to external stimuli such as carcinogens, inflammatory cytokines and
reactive oxygen species leads to increased nuclear translocation and DNA binding of NF-κB. Our results show that the animals of group II exhibit high expression of NF-κB and P38 MAP kinase as compared to control group (Group I). However, treatment of rats with Fagonia cretica/Carum carvi/diosmin abrogated the expression of NF-κB and P38 MAP kinase and therefore we suggest that these may be important not only in alleviating liver inflammation but also for the prevention of liver cancer.

Moreover, it has been demonstrated that the higher rate of P53 expression is associated with the higher degree of hepatocellular carcinoma malignancy (Nakano et al., 2003). Liver sections of rats of group II showed significant increase in P53 protein, which was reduced by the supplementation of Fagonia cretica/Carum carvi/diosmin. This might be attributed to the anti-mutagenic effect of Fagonia cretica/Carum carvi/diosmin which minimized DNA damage caused by DEN and 2-AAF. Another plausible explanation is that Fagonia cretica/Carum carvi/diosmin might have prevented high levels of wild-type p53, produced in response to Fagonia cretica/Carum carvi/diosmin, from being transformed into mutant p53 (Moon et al., 2008). The wild-type p53 is a tumor suppressor gene involved in several different mechanisms including gene transcription, DNA synthesis, repair, and programmed cell death (Kastan et al., 1991; Lane, 1992; Vogelstein and Kinzler, 1992; Harris and Hollstein, 1993; Wang and Liu, 2006) (38-42). Mutation in and loss of the p53 gene are the most common genetic defects in human malignant tumors, including hepatocellular carcinoma (Vogelstein and Kinzler, 1992; Anzola and Burgos, 2003).

In summary, the results of our present investigation demonstrate that modulators like Fagonia cretica, Carum carvi and diosmin exerts a striking chemopreventive effect against experimentally induced in vivo hepatocarcinogenesis in rats. The dose-responsive chemopreventive properties of these modulators (Fagonia cretica, Carum carvi and diosmin) have been reflected in their ability to abrogate the development of preneoplastic hepatic nodule formation. Our study also demonstrates that inhibition of cell proliferation and down regulation of inflammatory markers may be, at least in part, the underlying mechanisms related to the liver tumor inhibition by these modulators. Further studies are warranted to identify and isolate the major bioactive constituents present in Fagonia cretica and Carum carvi and to delineate their mechanisms of action responsible for inhibition of hepatic tumorigenesis. Nevertheless, the data presented here clearly encourage the development of Fagonia cretica, Carum carvi and diosmin phytochemicals for chemoprevention of human liver cancer.