Chapter VI

Attenuation of 2-Acetylaminofluorene induced early carcinogenic events by *Fagonia cretica*, *Carum carvi* and Diosmin
1. Introduction

There are several lines of evidence from both preclinical and clinical studies that support that oxidative stress caused by reactive oxygen species (ROS) plays a crucial role in the pathophysiology associated with various diseases such as, atherosclerosis, neurodegenerative diseases, and all stages of carcinogenesis (Scandalios, 2005). These ROS include free radicals such as hydroxyl radical, peroxy radical, superoxide anion radical, and other reactive species, such as hydrogen peroxide and singlet oxygen, generated as a result of naturally occurring processes (e.g., mitochondrial electron transport, exercise), environmental stimuli (e.g., ionizing radiation from the sun), environmental pollutants, changed atmospheric conditions (e.g., hypoxia), and lifestyle stressors (e.g., cigarette smoke and excess alcohol consumption). Generation of excessive ROS in cells lead to the imbalance in the oxidant and antioxidant levels and are responsible for the alterations in the normal homeostasis in the cell functioning. Hepatocellular carcinoma (HCC) is one of the most frequent tumors, with 0.25–1 million of newly diagnosed cases each year worldwide (Bruix et al., 2004; El-Serag and Rudolph, 2007; Llovet et al., 2003). The geographic distribution of HCC is highly uneven, with well recognised but various risk factors, such as male sex, increasing age, viral infection, cirrhosis, etc., in most regions about 80% of liver cancer occur in the presence of cirrhosis due to liver injury (Shikata, 1976; Okuda, 2000). Over the past decade, there is a growing interest in evaluating the chemopreventive strategies in encouraging the use of medicinal herbs and phyto-products against various health disorders including cancer. Experimental research has confirmed that the natural products and phytochemicals are associated with decreased cancer incidence (Stoner and Morse, 1996; Cremonezzi, 2001). The chemopreventive efficacy of these nutraceuticals has been attributed to the presence of high content of antioxidants such as flavinoids, polyphenols, terpenes etc. These potential agents can either abolish or delay the development of cancer by interfering with one or more steps in the process of carcinogenesis such as preventing the activation of carcinogen, by increasing detoxification or by blocking the interaction of ultimate
carcinogen with cellular macromolecules, or by suppressing the clonal expansion of neoplastic cells (Morse et al., 1993; Tanaka, 1994). Moreover, these chemopreventive agents can be targeted for intervention at either the initiation, promotion or progression stage of carcinogenesis (Stoner and Mukhtar, 1995; Morse and stoner, 1993).

Numerous agents determined to be safe and effective in preclinical trials have been and continue to be tested in clinical interventions for cancers at various sites including breasts, colon, prostate, oesophagus, mouth, lung, cervix, endometrium, ovary, liver, bladder and skin (Li et al., 2002). The intervention of cancer at the promotion stage, however, seems to be most appropriate and practical. The major reason for that relates to the fact that tumour promotion is a reversible event in early stages and requires repeated and prolonged exposure of promoting agents (Digiovanni, 1992). For this reason, it is important to identify antitumor and antipromoting agent present in the diets consumed by the human population.

Aromatic amines such as 2-acetylaminofluorene (2-AAF) have been shown to be potent tumorigenic agents in several tissue types (Miller, 1978). The administration of the carcinogen 2-AAF to rats has been widely used as an experimental model in the study of the series of events which comprise hepatocellular carcinogenesis (Emmelot, 1980; Schulte-Hermann, 1985; Sell et al., 1987; Farber and Sarma, 1985; Yuspa and Poirier, 1988). In vivo modification of 2-AAF to electrophilic metabolites and subsequent formation of covalent DNA adducts are believed to be essential steps in hepatocarcinogenesis (Miller, 1978). 2-AAF or its N-hydroxyl derivative forms three guanine adducts (Beland et al., 1982; Poirier, et al., 1982; Gupta, et al., 1982) and one tentatively identified adenine adduct (Gupta and Dighe, 1984) in rat liver DNA in vivo namely, N-acetyl-N-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AAF), 3-deoxyguanosin-N2-yl)-2-acetylamino-fluorene (dG-N2-AAF), N-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AAF), and N-acetyl-N-(deoxyadenosin-8-yl)-2-amino-fluorene. Formation of the predominant liver DNA adduct dG-C8-AAF has been shown to be directly related to in vivo mutagenesis (Beranek
et al., 1982). Moreover, reports from our laboratory and others have also confirmed that oxidative stress and DNA adduct formation to be the main pillars of 2-AAF induced tumor promotion response (Hillesheim et al., 1995; Rahman and Sultana, 2007; Khan and Sultana, 2011). Administration of 2-AAF promotes hepatocarcinogenesis which causes the loss of lipid peroxidation in preneoplastic hepatocellular membranes as of result of loss of mechanism of self control in cell proliferation (Benedetti et al., 1984). 2-AAF thus profoundly interferes with the in vivo regulation of several proteins essential for normal cell cycle progression. Oxidative stress is considered as an early event of carcinogenesis along with ornithine decarboxylase (ODC), PCNA, Ki67 induction and enhanced DNA synthesis. In various models of carcinogenesis, carcinogen induces ODC, PCNA and Ki67 and inhibitors of these proteins suppress cancer development (Auvinen, 1997; Khan et al., 2001, 2003, Ahmad et al., 2001; Zhen et al., 2006; Rehman and Sultana, 2011)

The aim of the present study is to assess the prognostic value of early tumor markers (Ki67, PCNA and ODC), oxidative DNA damage, apoptosis and oxidative stress in rat model induced by 2-acetylamino fluorene (2-AAF) and their amelioration by the administration of *Fagonia cretica/ Carum carvi/diosmin*. These modulators are known to possess various medicinal uses. *Fagonia cretica* has been reported to contain wide variety of antioxidants and triterpenoids saponins (Miyase et al., 1996; Khalik et al., 2000) and has been used for the treatment of fever, thirst, vomiting, dysentery, asthma, urinary discharges, liver trouble, typhoid, toothache, stomach troubles, stomatitis and skin diseases (Baquar, 1989). *Carum carvi* is known to possess, antidyseptic (Holtmann et al., 2003), antispasmodic (Eddouks et al., 2004), antiulcerogenic (Khayyal et al., 2001), antibacterial (Singh et al., 2002), antitumor (Kamaleeswari et al., 2006), antiproliferative (Nakano et al., 1998), antioxidant (Kamaleeswari et al., 2006), antihyperglycemic (Eddouks et al., 2004), antihyperlipidaemic (Lemhadri et al., 2006) and diuretic (Lahlou et al., 2007) activities. Diosmin (Diosmetin-7-O-rutinoside), a naturally occurring flavones glycoside has various biological activities including antioxidant activity (Cotelle et al., 1996), anti-
inflammatory effect (Crespo et al., 1999), anti-diabetic effect (Manuel et al., 1999) and anti-proliferative and anti-cancer activities (Tanaka et al., 1997). Moreover, diosmin has been found to increase the venous tone, improves lymphatic drainage and reduces the capillary hyperpermeability, thereby, leading to reduction in the release of inflammatory mediators (Lyseng-Williamson and Perry, 2003).
2. Treatment regimen for alleviation of 2-acetylaminofluorine (2-AAF) induced early tumor markers by *Fagonia cretica*, *Carum carvi* and Diosmin study

In this study, twenty four female Wistar rats were randomly allocated to 4 groups of 6 rats each. Group I as control, fed on normal diet. Groups II, III and IV were given 2-acetylaminofluorine (2-AAF) 0.02% w/w in diet for 14 days. Groups III and IV received modulator (*Fagonia cretica*, *Carum carvi* and Diosmin) at doses D1 & D2 respectively by gavage once daily for 14 days. On 7\textsuperscript{th} day, partial hepatectomy (PH) was done in the rats of groups II, III and IV. After 14 days, the animals were sacrificed by cervical dislocation under mild anaesthesia. Blood and liver samples were taken for various serum, biochemical and molecular parameters.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment regimen (14 days)</th>
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<tbody>
<tr>
<td>Group I</td>
<td>Normal Diet</td>
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<tr>
<td>Group II</td>
<td>2-AAF (0.02% in diet) + Partial Hepatectomy (PH) (on 7\textsuperscript{th} day)</td>
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<tr>
<td>Group III</td>
<td>2-AAF (0.02% in diet) + PH (on 7\textsuperscript{th} day) + Modulator (Low dose) D1</td>
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<tr>
<td>Group IV</td>
<td>2-AAF (0.02% in diet) + PH (on 7\textsuperscript{th} day) + Modulator (High dose) D2</td>
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</table>
3. Results

3.1. Study 1: Chemopreventive efficacy of *Fagonia cretica* against 2-acetylaminofluorene induced (2-AAF) early tumor markers and possible role of Caspases

3.1.1. Effect of administration of methanolic extract of *Fagonia cretica* on cytochrome P450 2E1 activity

2-AAF administration caused significant induction of CYP 2E1 (*p*<0.001) in hepatic tissues (25.47 ± 1.10) as compared with control group (15.09 ± 0.76). Treatment with *Fagonia cretica* brought back the level of CYP 2E1 to normal in hepatic tissues (D1= 19.69 ± 1.73, *p*<0.05, D2= 17.12 ± 1.67, *p*<0.01). (Table 1)

3.1.2. Effect of administration of methanolic extract of *Fagonia cretica* on Xanthine oxidase activity

Xanthine oxidase (XO) reflected significant increase (*p*<0.001) in the enzyme activity in hepatic (Table 1) tissue of ethanol treated group (1.06 ± 0.04) when compared with control (0.42 ± 0.31). *Fagonia cretica* significantly restores the level of xanthine oxidase (XO) activity (0.79 ± 0.04) & (0.47 ± 0.04) in liver tissue at dose D1 (*p*<0.01) and D2 (*p*<0.001) respectively.

3.1.3. Effect of administration of methanolic extract of *Fagonia cretica* on hepatic membrane damage (Lipid Peroxidation)

A significant (*p*<0.001) amplification of the MDA formation was found in 2-AAF treated group (Group II) in hepatic tissue (26.22 ± 1.59%) when compared with controls (10.49 ± 1.16). We have observed that treatment with *Fagonia cretica* at doses D1 and D2 leads to the significant restoration (*p*<0.01 and *p*<0.001 respectively) of membrane integrity in liver tissue (D1= 18.17 ± 1.72; D2= 12.05 ± 1.31) respectively when compared to 2-AAF treated group (Table 1).

3.1.4. *Fagonia cretica* treatment restores the activities of hepatic antioxidants

2-AAF treatment was found to diminish hepatic antioxidants GSH (0.039 ± 0.01, *p*<0.001), GPx (141.3 ± 9.76, *p*<0.001), GR (123.6 ± 27.38, *p*<0.01) and Catalase (10.21 ± 0.97, *p*<0.001) as compared to corresponding control group (GSH: 0.065 ± 0.01; GPx: 224.8 ± 12.4; GR: 291.4 ± 30.79 and Catalase: 21.6 ± 1.23). Treatment of *Fagonia cretica* significantly increases the level of GSH (D1: 0.055 ± 0.004, *p*<0.05; D2: 0.064 ± 0.003, *p*<0.01), GPx (D1: 189.5 ± 14.18, *p*< 0.05; D2: 210.5 ± 7.47, *p*<0.01), GR (D1: 249.2 ± 29.0, *p*< 0.05; D2: 275.6 ± 31.4, *p*<0.01) and Catalase (D1: 16.02 ± 1.21, *p*< 0.01; D2: 18.91 ± 0.98, *p*<0.001) in liver respectively (Table 1). Which indicates antioxidant property of *Fagonia cretica* against 2-AAF induced oxidative stress.
3.1.5. *Fagonia cretica* treatment attenuates 2-AAF induced hepatotoxicity

Group II showed 141%, 123% & 75% increase in serum AST, ALT and LDH levels respectively. Administration with *Fagonia cretica* was found effective in the normalization in these serum toxicity markers by 71% (p<0.01), 56% (p<0.001) & 44% (p<0.05) at D1 and 124% (p<0.001), 95% (p<0.001) & 72% (p<0.001) at dose D2 when compared to toxicant group (Table 1).

3.1.6. Effect of *Fagonia cretica* treatment on hepatic TNF-α level

TNF-α levels in the hepatic tissue of rats of group II was significantly elevated by 78% (p<0.001) as compared with control group. *Fagonia cretica* administration showed a significant reduction in the hepatic TNF-α levels by 61% at doses D1 (p<0.01) and by 37% at dose D2 (p<0.001) as compared with the group II (Figure 3).

3.1.7. Effect of *Fagonia cretica* administration on caspase 3, 7 and 9 activities

Figure 4 shows the expression of caspase 3, 7 and 9 in liver tissue of rats. There is a significant elevation of caspase activities in group II as compared with group I, where as both, groups III and IV showed a significant restoration of caspase activities (p<0.001) as compared to group II, confirming anti-apoptotic efficacy of *Fagonia cretica*.

3.1.8. Expression of proliferation markers (PCNA and Ki67)

Hepatic expressions of PCNA and Ki67 have been shown in the figures 1 and 2 respectively. In group II, there was higher number of cells showing expression of these proteins as indicated by the brown stains. Expression of these proliferation markers is markedly suppressed in the *Fagonia cretica* treated groups. For immunohistochemical analysis, brown colour indicates specific immune-staining of these proteins and light blue colour indicates haematoxylin staining (original magnification: 400x).

3.1.9. Histopathology of Liver tissue

Analysis of tissue sections of animals from different treatment groups under microscope (400 x magnifications) revealed marked changes when compared with control group animals (Fig. 5). In the animals of group II, there was an evident cellular proliferation and vacuolar degeneration around the central vein. Moreover 2-AAF causes apparent inflammatory response around the central vein in terms of infiltration of inflammatory cells (Fig.5B) in liver tissue. In contrast, *Fagonia cretica* administration at both the doses (100 and 200 mg/kg b. wt) protected the liver histology against 2-AAF induced alterations (Fig. 5C and D).
Table 1: Oxidative stress and cytotoxicity markers of liver toxicity and modulation by *Fagonia cretica*

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>2-AAF + PH</th>
<th><em>Fagonia cretica</em> D1 + 2-AAF + PH</th>
<th><em>Fagonia cretica</em> D2 + 2-AAF + PH</th>
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<tbody>
<tr>
<td><strong>CYP 2E1</strong> (nmol/mg protein)</td>
<td>15.09 ± 0.76</td>
<td>25.47 ± 1.1(^a)</td>
<td>19.69 ± 1.73(^*)</td>
<td>17.12 ± 1.67(^**)</td>
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<tr>
<td><strong>LPO</strong> (nmolMDA formed/g tissue)</td>
<td>10.49 ± 1.16</td>
<td>26.22 ± 1.59(^a)</td>
<td>18.17 ± 1.72(^**)</td>
<td>12.05 ± 1.31(^***)</td>
</tr>
<tr>
<td><strong>Catalase</strong> (nmol H(_2)O(_2) consumed/min/mg protein)</td>
<td>21.60 ± 1.23</td>
<td>10.21 ± 0.97(^*)</td>
<td>16.02 ± 1.21(^**)</td>
<td>18.91 ± 0.98(^**)</td>
</tr>
<tr>
<td><strong>Glutathione Peroxidase</strong> (nmol NADPH oxidized/min/mg protein)</td>
<td>224.8 ± 12.4</td>
<td>141.3 ± 9.76(^a)</td>
<td>189.5 ± 14.18(^*)</td>
<td>210.5 ± 7.47(^**)</td>
</tr>
<tr>
<td><strong>Glutathione reductase</strong> (nmol NADPH oxidized/min/mg protein)</td>
<td>291.4 ± 30.79</td>
<td>123.6 ± 27.38(^b)</td>
<td>248.2 ± 29.0(^*)</td>
<td>275.6 ± 31.38(^**)</td>
</tr>
<tr>
<td><strong>Reduced Glutathione</strong> (µmol GSH conjugate/g tissue) (+: SD)</td>
<td>0.065 ± 0.001(^+)</td>
<td>0.039 ± 0.001(^a)</td>
<td>0.055 ± 0.0037(^*)</td>
<td>0.064 ± 0.003(^**)</td>
</tr>
<tr>
<td><strong>H(_2)O(_2) Content</strong> (nM H(_2)O(_2)/min/mg tissue)</td>
<td>340.0 ± 23.32</td>
<td>570.2 ± 20.35(^a)</td>
<td>452.5 ± 20.46(^**)</td>
<td>395.5 ± 27.81(^***)</td>
</tr>
<tr>
<td><strong>Xanthine oxidase</strong> (µg of uric acid formed/min/mg protein)</td>
<td>0.42 ± 0.031</td>
<td>1.06 ± 0.04(^a)</td>
<td>0.79 ± 0.04(^**)</td>
<td>0.47 ± 0.04(^**)</td>
</tr>
<tr>
<td><strong>Aspartate aminotransferase</strong> (AST or SGOT) (IU/L)</td>
<td>27.21 ± 1.21</td>
<td>65.44 ± 3.46(^a)</td>
<td>46.04 ± 4.5(^**)</td>
<td>31.60 ± 3.27(^***)</td>
</tr>
<tr>
<td><strong>Alanine aminotransferase</strong> (ALT or SGPT) (IU/L)</td>
<td>13.02 ± 0.31</td>
<td>29.07 ± 0.81(^a)</td>
<td>21.73 ± 0.61(^**)</td>
<td>16.72 ± 0.56(^**)</td>
</tr>
<tr>
<td><strong>Lactate dehydrogenase</strong> (LDH) (nmol NADH oxidised/min/mg protein)</td>
<td>256.6 ± 27.0</td>
<td>449.7 ± 30.57(^a)</td>
<td>337.0 ± 25.87(^*)</td>
<td>264.5 ± 27.99(^**)</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.E., n = 6

\(^a\) P < 0.001, \(^b\) P<0.01 compared with the corresponding value for control group.

*P <0.05, **P <0.01 and ***P <0.001 compared with the corresponding value for toxicant group.

*Fagonia cretica* D1: 100mg/kg b. wt.; *Fagonia cretica* D2: 200mg/kg b. wt.
Figure 1: Effect of *Fagonia cretica* on PCNA expression in liver tissue

Photomicrograph represents the effect of Diosmin treatment on PCNA expressions. Brown color indicates specific immunostaining of PCNA, and light colour indicates haematoxylin staining. **A)** Control liver. **B)** 2-AAF + PH group. **C)** 2-AAF + PH + *Fagonia cretica* D1. **D)** 2-AAF + PH + *Fagonia cretica* D2. (400x)

*Fagonia cretica* D1: 100mg/kg b. wt.; *Fagonia cretica* D2: 200mg/kg b. wt.
Figure 2: Effect of *Fagonia cretica* on Ki67 expression in liver tissue

Photomicrograph represents the effect of Diosmin treatment on Ki67 expressions. Brown color indicates specific immunostaining of Ki67, and light colour indicates haematoxylin staining.

**A)** Control liver. **B)** 2-AAF + PH group. **C)** 2-AAF + PH + *Fagonia cretica* D1. **D)** 2-AAF + PH + *Fagonia cretica* D2. (400x)

*Fagonia cretica* D1: 100mg/kg b. wt.; *Fagonia cretica* D2: 200mg/kg b. wt.
Figure 3: Effect of *Fagonia cretica* on TNF-α level in rat liver

Graph showing effect of *Fagonia cretica* on 2-AAF induced TNF-α level in liver tissue

Each value represents mean ± S.E., n = 6

### P < 0.001, compared with the corresponding value for control group.

*P < 0.05, **P < 0.01 and ***P < 0.001 compared with the corresponding value for only toxicant treated group.

D1: *Fagonia cretica* 100mg/kg b. wt.; D2: *Fagonia cretica* 200mg/kg b. wt.
Figure 4: Effect of *Fagonia cretica* on Caspase 3, 7 and 9 activities in rat liver

Graph showing effect of *Fagonia cretica* on 2-AAF induced apoptosis in liver tissue.
Each value represents mean ± S.E., n = 6

### P < 0.001, compared with the corresponding value for control group.

*P <0.05, **P <0.01 and ***P <0.001 compared with the corresponding value for only toxicant treated group.

D1: *Fagonia cretica* 100mg/kg b. wt.; D2: *Fagonia cretica* 200mg/kg b. wt.
Figure 5: Effect of *Fagonia cretica* on histopathology of liver tissue

Photomicrograph showing the histopathological architecture of liver tissue of rats treated with *Fagonia cretica*

**A)** Control liver. **B)** 2-AAF + PH group. **C)** 2-AAF + PH + *Fagonia cretica* D1. **D)** 2-AAF + PH + *Fagonia cretica* D2. (400x)

D1: *Fagonia cretica* 100mg/kg b. wt.; D2: *Fagonia cretica* 200mg/kg b. wt.
3.2. Study 2: Evaluation of beneficial effects of *Carum carvi* on early tumour markers and apoptosis/necrosis.

3.2.1. Effect of administration of methanolic extract of *Carum carvi* on cytochrome P450 2E1 activity

2-AAF administration caused significant induction of CYP 2E1 (p<0.001) in hepatic tissues (25.47 ± 1.10) as compared with control group (15.09 ± 0.76). Treatment with *Carum carvi* brought back the level of CYP 2E1 to normal in hepatic tissues (D1 = 20.37 ± 1.8, p<0.05, D2 = 17.12 ± 1.67, p<0.01). (Table 2)

3.2.2. Effect of administration of methanolic extract of *Carum carvi* on Xanthine oxidase activity

Xanthine oxidase (XO) reflected significant increase (p<0.001) in the enzyme activity in hepatic (Table 2) tissue of ethanol treated group (1.06 ± 0.04) when compared with control (0.42 ± 0.31). *Carum carvi* significantly restores the level of xanthine oxidase (XO) activity (0.81 ± 0.03) & (0.59 ± 0.05) in liver tissue at dose D1 (p<0.01) and D2 (p<0.001) respectively.

3.2.3. Effect of administration of methanolic extract of *Carum carvi* on hepatic membrane damage (Lipid Peroxidation)

A significant (p<0.001) amplification of the MDA formation was found in 2-AAF treated group (Group II) in hepatic tissue (26.22 ± 1.59%) when compared with controls (10.49 ± 1.16). We have observed that treatment with *Carum carvi* at doses D1 and D2 leads to the significant restoration (p<0.01 and p<0.001 respectively) of membrane integrity in liver tissue (D1 = 18.61 ± 1.13; D2 = 12.47 ± 1.36) respectively when compared to 2-AAF treated group (Table 2).

3.2.4. *Carum carvi* treatment restores the activities of hepatic antioxidants

2-AAF treatment was found to diminish hepatic antioxidants GSH (0.039 ± 0.01, p<0.001), GPx (141.3 ± 9.76, p<0.001), GR (123.6 ± 27.38, p<0.01) and Catalase (10.21 ± 0.97, p<0.001) activities as compared to corresponding control group (GSH: 0.065 ± 0.01; GPx: 224.8 ± 12.4; GR: 291.4 ± 30.79 and Catalase: 21.6 ± 1.23) respectively. Treatment of *Carum carvi* significantly increases the level of GSH (D1: 0.052 ± 0.008, p=NS; D2: 0.058 ± 0.009, P<0.05), GPx (D1: 171.1 ± 12.57, p= NS; D2: 185.6 ± 8.59, p<0.05), GR (D1: 257.8 ± 28.89, p< 0.05; D2: 285.8 ± 37.41, p<0.01) and Catalase (D1: 13.85 ± 0.7, p=NS; D2: 15.14 ± 1.07, p<0.05) in liver respectively as compared with group II (Table 2). This indicates antioxidant property of *Carum carvi* against 2-AAF induced oxidative stress.
3.2.5. **Effect of *Carum carvi* on H$_2$O$_2$ content in liver tissue of rats of all groups**

It has been observed that rats of group II showed a significant increase in the H$_2$O$_2$ content as compared with control group. Treatment with *Carum carvi* resulted in the restoration of H$_2$O$_2$ content at both doses D1 (p<0.05) and D2 (p<0.001). (Table 2)

3.2.6. **Carum carvi** treatment attenuates 2-AAF induced hepatotoxicity

Group II showed 141%, 123% & 75% increase in serum AST, ALT and LDH levels respectively as compared with control group (Group I). Administration with *Carum carvi* was found effective in the normalization in these serum toxicity markers by 65% (p<0.01), 32% (p<0.01) & 44% (p<0.05) at dose D1 and 119% (p<0.001), 92% (p<0.001) & 66% (p<0.01) at dose D2 when compared to group II (Table 2).

3.2.7. **Effect of *Carum carvi* treatment on hepatic TNF-$\alpha$ level**

TNF-$\alpha$ levels in the hepatic tissue of rats of group II was significantly elevated by 78% (p<0.001) as compared with control group. *Carum carvi* administration showed a significant reduction in the hepatic TNF-$\alpha$ levels by 54% at doses D1 (p<0.05) and by 71% at dose D2 (p<0.01) as compared with the group II (Figure 6).

3.2.8. **Effect of *Carum carvi* administration on caspase 3, 7 and 9 activities**

Figure 7 shows the expression of caspase 3, 7 and 9 in liver tissue of rats. There is a significant elevation of caspase activities in group II as compared with group I, where as *Carum carvi* at both doses (groups III and IV) showed a significant restoration of caspase activities (p<0.001) as compared to group II, confirming anti-apoptotic efficacy of *Carum carvi*.

3.2.9. **Expression of proliferation marker (PCNA)**

Hepatic expressions of PCNA have been shown in the figure 8. In group II, there was higher number of cells showing expression of these proteins as indicated by the brown stains. Expression of these proliferation markers is markedly suppressed in the *Carum carvi* treated groups. (Figure 8) For immunohistochemical analysis, brown colour indicates specific immune-staining of these proteins and light blue colour indicates haematoxylin staining (original magnification: 400x).

3.2.10. **Histopathology of Liver tissue**

Analysis of tissue sections of animals from different treatment groups under microscope (400x magnification) revealed marked changes when compared with control group animals (Fig. 9). In
the animals of group II, there was an evident cellular proliferation and vacuolar degeneration around the central vein. Moreover 2-AAF causes apparent inflammatory response around the central vein in terms of infiltration of inflammatory cells (Fig. 9B) in liver tissue. In contrast, Carum carvi administration at both the doses (100 and 200 mg/kg b. wt) protected the liver histology against 2-AAF induced alterations (Fig. 9C and D).
Table 2: Oxidative stress and cytotoxicity markers of liver toxicity and modulation by *Carum carvi*

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>2-AAF + PH</th>
<th>Carum carvi D1 + 2-AAF + PH</th>
<th>Carum carvi D2 + 2-AAF + PH</th>
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<tr>
<td><strong>CYP 2E1</strong> (nmol/mg protein)</td>
<td>15.09 ± 0.76</td>
<td>28.47 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.48 ± 0.99**</td>
<td>16.43 ± 1.01***</td>
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<td><strong>LPO</strong> (nmol MDA formed/g tissue)</td>
<td>10.49 ± 1.16</td>
<td>26.22 ± 1.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.61 ± 1.13**</td>
<td>12.47 ± 1.36***</td>
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<tr>
<td><strong>Catalase</strong> (nmol H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt; consumed/min/mg protein)</td>
<td>21.60 ± 1.23</td>
<td>10.21 ± 0.97&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.85 ± 0.7&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>15.14 ± 1.07&lt;sup&gt;*&lt;/sup&gt;</td>
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<tr>
<td><strong>Glutathione Peroxidase</strong> (nmol NADPH oxidized/min/mg protein)</td>
<td>224.8 ± 12.4</td>
<td>141.3 ± 9.76&lt;sup&gt;a&lt;/sup&gt;</td>
<td>171.1 ± 12.57&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>185.6 ± 8.59&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Glutathione reductase</strong> (nmol NADPH oxidized/min/mg protein)</td>
<td>291.4 ± 30.79</td>
<td>123.6 ± 27.38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>257.8 ± 28.89&lt;sup&gt;*&lt;/sup&gt;</td>
<td>285.8 ± 37.41**</td>
</tr>
<tr>
<td><strong>Reduced Glutathione</strong> (µmol GSH conjugate/g tissue) (+ : SD)</td>
<td>0.065 ± 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.039 ± 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.052 ± 0.008&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>0.058 ± 0.009&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt; Content</strong> (nM H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;/min/mg tissue)</td>
<td>340.0 ± 23.32</td>
<td>570.2 ± 20.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>486.7 ± 20.34&lt;sup&gt;*&lt;/sup&gt;</td>
<td>437.5 ± 20.06**</td>
</tr>
<tr>
<td><strong>Xanthine oxidase</strong> (µg of uric acid formed/min/mg protein)</td>
<td>0.42 ± 0.03</td>
<td>1.06 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.8 ± 0.03**</td>
<td>0.59 ± 0.05***</td>
</tr>
<tr>
<td><strong>Aspartate aminotransferase</strong> (AST or SGOT) (IU/L)</td>
<td>27.21 ± 1.21</td>
<td>65.44 ± 3.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.63 ± 4.81**</td>
<td>33.17 ± 3.66***</td>
</tr>
<tr>
<td><strong>Alanine aminotransferase</strong> (ALT or SGPT) (IU/L)</td>
<td>13.02 ± 0.31</td>
<td>29.07 ± 0.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.85 ± 1.06**</td>
<td>17.08 ± 0.42***</td>
</tr>
<tr>
<td><strong>Lactate dehydrogenase</strong> (LDH) (nmol NADH oxidised/min/mg protein)</td>
<td>256.6 ± 27.0</td>
<td>449.7 ± 30.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>346.49 ± 25.72&lt;sup&gt;*&lt;/sup&gt;</td>
<td>280.37 ± 25.8**</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.E., n = 6

<sup>a</sup>P < 0.001 , <sup>b</sup>P < 0.01 compared with the corresponding value for control group.

*P < 0.05, **P < 0.01 and ***P < 0.001 compared with the corresponding value for group II.

*Carum carvi* D1 and D2 represents the dose of *Carum carvi* at 100 and 200 mg/kg b. wt.
Figure 6: Effect of *Carum carvi* on TNF-α level in hepatic tissue

Representative graph showing the induction of TNF-α by 2-AAF administration and its modulation by *Carum carvi*.

Each value represents mean ± S.E., $n = 6$

### $P < 0.001$, compared with the corresponding value for control group.

* $P < 0.05$, ** $P < 0.01$ compared with the corresponding value for only toxicant treated group.

*Carum carvi* D1 and D2 represents the dose of *Carum carvi* at 100 and 200mg/kg b. wt.
Figure 7: Effect of treatment of *Carum carvi* on Caspase activities in liver tissue

Representative graphs showing the effect of *Carum carvi* treatment on 2-AAF induced apoptosis in rat liver.

Each value represents mean ± S.E., n = 6

### P < 0.001, compared with the corresponding value for control group.

**P <0.01 and ***P <0.001 compared with the corresponding value for only toxicant treated group.

*Carum carvi* D1 and D2 represents the dose of *Carum carvi* at 100 and 200mg/kg b. wt.
Figure 8: Effect of treatment of *Carum carvi* on PCNA expression in liver tissue

Photomicrograph represents the effect of Diosmin treatment on PCNA expressions. Brown color indicates specific immunostaining of PCNA, and light colour indicates haematoxylin staining. **A)** Control liver. **B)** 2-AAF + PH group. **C)** 2-AAF + PH + *Carum carvi* D1. **D)** 2-AAF + PH + *Carum carvi* D2. (Original magnification 400x)

*Carum carvi* D1 and D2 represents the dose of *Carum carvi* at 100 and 200mg/kg b. wt.
Figure 9: Effect of treatment of *Carum carvi* on histopathology of liver tissue

Photomicrograph represents the histopathological changes in the liver tissues of rats. **A)** Control liver. **B)** 2-AAF + PH group. **C)** 2-AAF + PH + *Carum carvi* D1. **D)** 2-AAF + PH + *Carum carvi* D2. (400x)

*Carum carvi* D1 and D2 represents the dose of *Carum carvi* at 100 and 200mg/kg b. wt.
3.3. Study 3: Evaluation of beneficial effects of diosmin on early tumour markers and apoptosis/necrosis.

3.3.1. Effect of administration of methanolic extract of diosmin on cytochrome P450 2E1 activity

2-AAF administration caused significant induction of CYP 2E1 (p<0.001) in hepatic tissues (25.47 ± 1.10) as compared with control group (15.09 ± 0.76). Treatment with diosmin brought back the level of CYP 2E1 to normal in hepatic tissues (D1= 19.48 ± 0.99, p<0.01, D2= 16.43 ± 1.01, p<0.01). (Table 3)

3.3.2. Effect of administration of methanolic extract of diosmin on Xanthine oxidase activity

Xanthine oxidase (XO) reflected significant increase (p<0.001) in the enzyme activity in hepatic (Table 3) tissue of animals of group II (1.06 ± 0.04) when compared with control (0.42 ± 0.31). Diosmin significantly restores the level of xanthine oxidase (XO) activity (0.69 ± 0.04) & (0.47 ± 0.04) in liver tissue at dose D1 (p<0.001) and D2 (p<0.001) respectively.

3.3.3. Effect of administration of diosmin on hepatic membrane damage (Lipid Peroxidation)

A significant (p<0.001) amplification of the MDA formation was found in 2-AAF treated group (Group II) in hepatic tissue (26.22 ± 1.59) when compared with controls (10.49 ± 1.16). We have observed that treatment with Diosmin at doses D1 and D2 leads to the significant restoration (p<0.05 and p<0.01) of membrane integrity in liver tissue (D1= 19.48 ± 1.93; D2= 17.11 ± 1.47) respectively when compared to 2-AAF treated group (Table 3).

3.3.4. Diosmin treatment restores the activities of hepatic antioxidants

2-AAF treatment was found to diminish hepatic antioxidants GSH (0.039 ± 0.01, p<0.001), GPx (141.3 ± 9.76, p<0.001), GR (123.6 ± 27.38, p<0.01) and Catalase (10.21 ± 0.97, p<0.001) as compared to corresponding control group (GSH: 0.065 ± 0.01; GPx: 224.8 ± 12.4; GR: 291.4 ± 30.79 and Catalase: 21.6 ± 1.23). Treatment of Diosmin significantly increases the level of GSH (D1: 0.057 ± 0.006, p<0.05; D2: 0.061 ± 0.009, P<0.01), GPx (D1: 191.9 ± 14.89, p<0.05; D2: 209.5 ± 9.67, p<0.01), GR (D1: 240.6 ± 26.22, p< 0.05; D2: 287.2 ± 31.49, p<0.01) and Catalase (D1: 17.09 ± 1.5, p<0.01; D2: 19.41 ± 1.52, p<0.001) in liver respectively (Table 3).
3.3.5. Effect of Diosmin on $\text{H}_2\text{O}_2$ content in liver tissue of rats

It has been observed that rats of group II showed a significant increase in the $\text{H}_2\text{O}_2$ content (570.2 ± 20.35, \(p<0.001\)) as compared with control group (340 ± 23.32). Treatment with Diosmin resulted in the restoration of $\text{H}_2\text{O}_2$ content at both doses D1 (381.5 ± 15.53, \(p< 0.001\)) and D2 (343.3 ± 16.37, \(p<0.001\)). (Table 3)

3.3.6. Diosmin treatment attenuates 2-AAF induced hepatotoxicity

Group II showed 141%, 123% & 75% increase in serum AST, ALT and LDH levels respectively as compared with control group (Group I). Administration with diosmin was found effective in the normalization in these serum toxicity markers by 62% \((p<0.001)\), 78% \((p<0.01)\) & 62% \((p<0.01)\) at D1 and 130% \((p<0.001)\), 114% \((p<0.001)\) & 73% \((p<0.001)\) at dose D2 respectively when compared to group II (Table 3).

3.3.7. Effect of Diosmin treatment on hepatic TNF-α level

TNF-α levels in the hepatic tissue of rats of group II was significantly elevated by 78% \((p<0.001)\) as compared with control group. Diosmin administration showed a significant reduction in the hepatic TNF-α levels by 58% at doses D1 \((p<0.01)\) and by 73% at dose D2 \((p<0.001)\) as compared with the group II (Figure 10).

3.3.8. Effect of Diosmin administration on caspase 3, 7 and 9 activities

Figure 11 shows the caspase activities in the hepatic tissue. There is a significant elevation of caspase activities in group II \((p<0.001)\) as compared with group I, where as Diosmin at both doses D1 and D2 (groups III and IV) showed a significant restoration of caspase activities \((p<0.001)\) as compared to group II, confirming anti-apoptotic efficacy of Diosmin.

3.3.9. Depletion of ornithine decarboxylase (ODC) activity by diosmin

2-AAF administration in group II resulted in 134% enhanced activity of early tumor marker, ornithine decarboxylase activity \((p<0.001)\) as compared with the control group (Group I). Diosmin treatment to rats in groups III and IV showed a significant depletion of ODC activity by 57% \((p<0.01)\) and 110% \((p<0.001)\) respectively as compared with group II. It indicates that diosmin possess a significant ODC suppressing efficacy. (Figure 12)

3.3.10. Effect of diosmin on DNA fragmentation in rat liver by 2-AAF administration

Figure 13 shows the DNA fragmentation of the rat liver DNA. It has been observed that the DNA isolated from the rats of group II showed DNA breakage as compared to the control
group (Group I). Conversely, diosmin administration prevents the 2-AAF induced DNA damage.

3.3.11. Expression of proliferation markers (PCNA and Ki67)

Figure 14 shows the PCNA and Ki67 expressions in liver tissues. It has been observed that hepatic tissues from animals of group II showed high expression of proliferation markers (PCNA and Ki67). Expression of these proliferation markers is markedly suppressed in the diosmin treated groups (Group III and IV). For immunohistochemical analysis, brown colour indicates specific immune-staining of these proteins and light blue colour indicates haematoxylin staining (original magnification: 400x).

3.3.12. Histopathology of Liver tissue

Analysis of tissue sections of animals from different treatment groups under microscope (400x magnification) revealed marked changes when compared with control group animals (Fig. 15). In the animals of group II, there was an area with cytoplasmic granulation with large hyperchromatic nuclei and vacuolar degeneration around the central vein in liver tissue (Fig.15B). In contrast, Diosmin administration at both the doses (10 and 20 mg/kg b. wt) protected the liver histology against 2-AAF induced alterations (Fig. 15C and D).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>2-AAF + PH</th>
<th>Diosmin D1 + 2-AAF + PH</th>
<th>Diosmin D2 + 2-AAF + PH</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP 2E1 (nmol/mg protein)</td>
<td>15.09 ± 0.76</td>
<td>25.47 ± 1.1 *</td>
<td>19.48 ± 0.99 **</td>
<td>16.43 ± 1.01 ***</td>
</tr>
<tr>
<td>LPO (nmol MDA formed/g tissue)</td>
<td>10.49 ± 1.16</td>
<td>26.22 ± 1.59 a</td>
<td>19.48 ± 1.93 *</td>
<td>17.11 ± 1.47 **</td>
</tr>
<tr>
<td>Catalase (nmol H$_2$O$_2$ consumed/min/mg protein)</td>
<td>21.60 ± 1.23</td>
<td>10.21 ± 0.97 a</td>
<td>17.09 ± 1.5 **</td>
<td>19.41 ± 1.52 ***</td>
</tr>
<tr>
<td>Glutathione Peroxidase (nmol NADPH oxidized/min/mg protein)</td>
<td>224.8 ± 12.4</td>
<td>141.3 ± 12.78 a</td>
<td>191.9 ± 14.89 *</td>
<td>209.5 ± 9.67 **</td>
</tr>
<tr>
<td>Glutathione reductase (nmol NADPH oxidized/min/mg protein)</td>
<td>291.4 ± 30.79</td>
<td>123.6 ± 27.38 b</td>
<td>240.6 ± 26.22 *</td>
<td>287.2 ± 31.49 **</td>
</tr>
<tr>
<td>Reduced Glutathione (µmol GSH conjugate/min/mg tissue)</td>
<td>0.065 ± 0.001 a</td>
<td>0.039 ± 0.001 a</td>
<td>0.056 ± 0.006 *</td>
<td>0.061 ± 0.009 **</td>
</tr>
<tr>
<td>H$_2$O$_2$ Content (nM H$_2$O$_2$/min/mg tissue)</td>
<td>340.0 ± 23.32</td>
<td>570.2 ± 20.35 *</td>
<td>381.5 ± 15.53 **</td>
<td>343.3 ± 16.37 ***</td>
</tr>
<tr>
<td>Xanthine oxidase (µg of uric acid formed/min/mg protein)</td>
<td>0.42 ± 0.03</td>
<td>1.06 ± 0.04 a</td>
<td>0.69 ± 0.04 **</td>
<td>0.47 ± 0.037 ***</td>
</tr>
<tr>
<td>Aspartate aminotransferase (AST or SGOT) (IU/L)</td>
<td>27.21 ± 1.21</td>
<td>65.44 ± 3.46 a</td>
<td>48.65 ± 2.03 **</td>
<td>29.95 ± 4.2 ***</td>
</tr>
<tr>
<td>Alanine aminotransferase (ALT or SGPT) (IU/L)</td>
<td>13.02 ± 0.31</td>
<td>29.07 ± 0.81 a</td>
<td>18.93 ± 1.88 **</td>
<td>14.17 ± 1.29 ***</td>
</tr>
<tr>
<td>Lactate dehydrogenase (LDH) (nmol NADH oxidised/min/mg protein)</td>
<td>256.6 ± 27.0</td>
<td>449.7 ± 30.57 a</td>
<td>290.8 ± 25.85 **</td>
<td>261.9 ± 30.6 ***</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.E., n = 6

* P < 0.001, **P < 0.01 compared with the corresponding value for control group.
*P < 0.05, **P < 0.01 and ***P < 0.001 compared with the corresponding value for only toxicant treated group. Diosmin D1: 10mg/kg b. wt and Diosmin D2: 20mg/kg b. wt.
Figure 10: Effect of diosmin on TNF-α expression in liver tissue

Each value represents mean ± S.E., n = 6

### P<0.001 compared with the corresponding value for control group.

** P<0.01, *** P <0.001 compared with the corresponding value for toxicant treated group.

Diosmin D1: 10mg/kg b. wt and Diosmin D2: 20mg/kg b. wt.

Figure 11: Effect of diosmin on Caspase 3, 7 and 9 activities

Each value represents mean ± S.E., n = 6

### P<0.001 compared with the corresponding value for control group.

*** P <0.001 compared with the corresponding value for toxicant treated group.

Diosmin D1: 10mg/kg b. wt and Diosmin D2: 20mg/kg b. wt.
Figure 12: Effect of diosmin on ornithine decarboxylase (ODC) activity

Each value represents mean ± S.E., n = 6
***P<0.001 compared with the corresponding value for control group.
**P<0.01, ***P<0.001 compared with the corresponding value for toxicant treated group.
Diosmin D1: 10mg/kg b. wt and Diosmin D2: 20mg/kg b. wt.

Figure 13: 2-AAF induced oxidative DNA fragmentation

C: Control group
T: 2-AAF + PH group
D1: 2-AAF + PH + Diosmin D1 Group
D2: 2-AAF + PH + Diosmin D2 Group
Diosmin D1: 10mg/kg b. wt and Diosmin D2: 20mg/kg b. wt.
Figure 14: Effect of diosmin treatment on and Ki67 (a) and PCNA (b) expressions

Fig. 14 (a): Ki67

Photomicrograph represents the effect of Diosmin treatment on Ki67 expressions. Brown color indicates specific immunostaining of Ki67, and light color indicates haematoxylin staining. A) Control liver. B) 2-AAF + PH group. C) 2-AAF + PH + Diosmin D1. D) 2-AAF + PH + Diosmin D2. (Original magnification 400x)
Diosmin D1: 10mg/kg b. wt and Diosmin D2: 20mg/kg b. wt.
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Attenuation of 2-AAF induced early tumour...

Figure 14 (b): PCNA expressions in liver tissue

Fig. 14 (b): PCNA

Photomicrograph represents the effect of Diosmin treatment on PCNA expressions. Brown color indicates specific immunostaining of PCNA, and light colour indicates haematoxylin staining. **A)** Control liver. **B)** 2-AAF + PH group. **C)** 2-AAF + PH + Diosmin D1. **D)** 2-AAF + PH + Diosmin D2. (Original magnification 400x)

Diosmin D1: 10mg/kg b. wt and Diosmin D2: 20mg/kg b. wt.
Figure 15: Effect of Diosmin on hepatic histopathology

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)** **Group II:** 2-AAF + PH group.

- **C)** **Group III:** 2-AAF + PH + Diosmin D1.

- **D)** **Group IV:** 2-AAF + PH + Diosmin D2. (H&E; original magnification ×400).

Diosmin D1: 10mg/kg b. wt and Diosmin D2: 20mg/kg b. wt.
4. Discussion

Natural compounds that suppress the activities of chemical activating enzymes might be good candidates for protecting against chemically induced toxicities. Chemically induced toxicities are often associated with the disturbance of normal prooxidant-antioxidant equilibrium in body that leads to oxidative stress, which is characterized by the increased generation of reactive oxygen species. Reactive oxygen species play an important role in many human degenerative diseases including cancer (Behrend et al., 2003; Apel and Hirt, 2004; Bergamini et al., 2004; Reddy and Clark, 2004; Shah and Channon, 2004; Willner, 2004; Cerutti, 1994). It has been demonstrated that CYP 2E1 is involved in the bioactivation of 2-Acetylaminofluorene (2-AAF) that is associated with centrilobular hepatic necrosis (Liu et al., 2005). Partial hepatectomy (PH) corresponds to the method of surgically inducing hyperplasia for speedy onset of early carcinogenic events (Best and Coleman, 2007; Park and Suh, 1999). In the present study, we have also observed that animals (group II), undergone partial hepatectomy besides 2-AAF administration show increased expressions of cell proliferation markers.

In the present study, we have observed the chemopreventive efficacy of *Fagonia cretica, Carum carvi* and diosmin against 2-AAF induced early proliferative events and hepatotoxicity in Wistar rats. All the modulators (*Fagonia cretica, Carum carvi* and diosmin) used in this study showed a significant recovery in the level of glutathione and its metabolizing enzyme in the liver. Moreover, these modulators induce the detoxifying enzyme system in the hepatic tissue, which is evident by the elevated levels of other important phase II enzymes (Catalase, GST, GR and GPxs). Lipid peroxidation (LPO) is one of the principal causes of chemically induced liver injury and is mediated by the production of free radicals. Treatment of animals with *Fagonia cretica/ Carum carvi*/diosmin dose dependently decreased the level of 2-AAF-induced lipid peroxides and \( \text{H}_2\text{O}_2 \) content. From the results obtained in the present study, it can be said that *Fagonia cretica/ Carum carvi*/diosmin exhibit powerful free radical scavenging activity, may therefore act by scavenging the reactive oxygen species formed during 2-AAF metabolism. Any compound,
natural or synthetic, with antioxidant properties that might contribute towards the partial or total alleviation of this damage, might have a significant role in maintaining good health when used as a medicine or consumed as a part of the normal diet.

Increased level of serum transaminases (AST and ALT) are the diagnostic markers of hepatic cellular damage because these enzymes are normally located in cytoplasm and are released into the circulation after hepatic damage (Recknagel, 1987; Recknagel et al., 1989). The methanolic extract of *Fagonia cretica* and *Carum carvi* and diosmin used in the present study seems to offer dose-dependent protection and maintain the structural integrity of hepatic cells. This was evident from the significant reduction in 2-AAF induced enhancement in serum AST, ALT and LDH activities. Moreover, we have also observed that short term administration of 2-AAF in diet to rats induces apoptosis and oxidative DNA damage. Induction of apoptosis is evident by the tremendous enhanced caspase activities in hepatic tissues in the rats of group II. Treatments with *Fagonia cretica*, *Carum carvi*, diosmin significantly prevent the apoptotic cell death and oxidative DNA damage. These investigations validate the use of *Fagonia cretica* and *Carum carvi* by Ayurvedic physicians in liver disorders.

Oxidative stress, Ornithine decarboxylase (ODC) induction, expression of PCNA and Ki67 are considered as prognostic events of carcinogenesis. In various models of chemical carcinogenesis, carcinogen induces ODC activity and inhibitors of ODC suppress cancer development. Previously reports from our laboratories also demonstrate the elevated activity of ODC by 2-AAF (Sehrawat and Sultana, 2006). Ornithine decarboxylase (ODC) induction has been associated with tumor promotion in skin (Boutwell, 1978) and liver (Olison and Russell, 1980), so that it might be a molecular marker for tumor promotion. ODC is the first and rate-limiting enzyme in polyamine biosynthesis (Raina et al., 1976) and has a very rapid turnover time of 10-20 min in liver (Russell et al., 1970; Russell and Snyder 1969). Drugs, hormones, and partial hepatectomy which increased cellular proliferation have been shown to increase ODC activity (Russell et al., 1976).
Increased ODC activity and expression PCNA and Ki67 are used extensively as a biochemical marker to evaluate the tumor promoting potential of an agent (Tarao et al., 1992; Ojanguren et al., 1993; De Riese et al., 1993; Barbareschi et al., 1994; Lin et al., 2000). In the present study, diosmin administration to rats significantly inhibits ODC activity. Moreover, expression of PCNA and Ki67 in the hepatic tissues has been evidently suppressed by the administration of Fagonia cretica, Carum carvi, diosmin. This significant decrease in the level of ODC activity by diosmin and suppression of PCNA and Ki67 by the treatment of Fagonia cretica/ Carum carvi/ diosmin suggests their role as antitumor promoting agents.

Our data, however, provide a substantial amount of mechanistic approach to show the chemopreventive effect of Fagonia cretica, Carum carvi, diosmin against oxidative stress, and tumor promotion related aspects of carcinogenicity. Hence, the present study clearly demonstrates the role of these modulators in the inhibition of biochemical events of tumor promotion.