Chapter IV

Amelioration of ethanol induced liver damage in Wistar rats: Promising role of *Fagonia cretica*, Chrysin and Diosmin

![Diagram of ethanol metabolism]

![Chemical structures of Fagonia cretica, Chrysin, Diosmin]
1. Introduction

Alcohol (Ethanol) is the main cause of end stage liver diseases worldwide. Alcohol related disorders are one of the current challenging health problems associated with the socio-economical consequences (Diehl, 2002). Alcohol consumption is associated with the toxicity to various organs of the body including liver, brain, kidney etc (Lieber, 1988). Oxidative stress is known to play an important role in the pathogenesis of ethanol-induced liver injury (Lindros, 1995; Rodrigo, et al., 1998 and Zima et al., 2001). Ethanol administration can elicit disturbances in the delicate balance between the pro- and antioxidant system of the body, therefore leading to oxidative stress. About 50 years ago, it was believed that ethanol is nontoxic and the actual cause for alcoholic liver disease is the nutritional deficiencies associated with alcohol consumption. But it was first time shown by Lieber and De Carle, that besides sufficient nutritional supplementation, rats develop hepatic injuries (Lieber and De Carli, 1967). The toxicity of ethanol was later on shown to be related to its metabolism by alcohol dehydrogenase (Zima et al., 2001). The first indications that not only alcohol dehydrogenase participate in the metabolism of ethanol came in the early 1970’s, when it was discovered that microsomal membrane fractions were capable of catalyzing the oxidation of ethanol. These reactions required NADPH, and were inhibited by CO as these properties were distinct from those of alcohol dehydrogenase (Lieber et al., 1970). It was then discovered that this activity was due to cytochrome P450 2E1 (CYP2E1) and that the enzyme was inducible by ethanol in rats (Ryan et al., 1986; Johansson et al., 1988). There is also a component of metabolism by catalase (Zima, 1993).

The main pathway for ethanol oxidation in the liver is via ADH to acetaldehyde, which is associated with the reduction of NAD to NADH. NADH in turn increases xanthine oxidase activity, which elevates production of superoxide (Zima et al., 2001). Metabolism of ethanol by alcohol dehydrogenase influences the redox status of the liver also in other ways. Enhanced acetaldehyde production after ethanol metabolism decreases hepatic glutathione (GSH) content. The decrease in GSH is both due to an increased loss, as well as a lower rate of synthesis (Speisky et al., 1985). The absolute majority of ethanol oxidation is by ADH to acetaldehyde and further by Aldehyde dehydrogenase to acetic acid. However, there is also a slight portion of P450 dependent inducible ethanol oxidation due to the CYP2E1 component. Also, CYP1A2, CYP3A4 and CYP2B families may contribute to ethanol oxidation (Johansson et al., 1988; Lieber, 2004).

Free radicals have been implicated in alcoholic liver disease in various ways. Mechanisms that are thought to be involved are impairment of antioxidant defences, as well as
production of reactive oxygen species by the mitochondria and the CYP2E1 enzyme, and by activated phagocytic cells. Oxidative compounds then may lead to activation of immune cells to express pro-fibrotic and pro-inflammatory cytokines. Macrophages produce TNF-\(\alpha\) in various conditions that cause oxidative stress (Ahmed et al., 2000), as well as IL-1 and IL-6 (Meng and Lowell, 1997). Also, oxidative stress leads to the generation of lipid peroxidation products and protein adducts (Dupont et al., 1998; Johansson and Ingelman-Sundberg, 1985; Albano, French et al. 1999), which eventually stimulate a break in self-tolerance and an immune reaction associated with hepatitis (Albano 2006). CYP2E1 inhibitors have been shown to reduce the formation of lipid peroxidation products (Ingelman-Sundberg et al., 1993).

It was suggested that CYP2E1 may play a role in alcoholic liver damage as it has been shown that during ethanol oxidation, CYP2E1 produces \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\) as a result of uncoupling of oxygen consumption with NADPH oxidation (Ingelman-Sundberg and Johansson 1984). In the presence of iron catalysts, even more reactive oxygen species (ROS) can be formed, such as the hydroxyl radical, superoxide and hydrogen peroxide (Ingelman-Sundberg and Johansson 1984; Gonzalez, 2005; Cederbaum, 2006). These reactive oxygen species can in turn lead to the elevated levels of lipid peroxidation products, that in turn lead to the adduct formation with the cellular macromolecules including nucleic acids and proteins and limit their normal functions. Eventually this will result in the cell damage (Cederbaum, 2006). It has been further demonstrated that, these elevated levels of ROS lead to the activation of immune response, which worsens as the disease progresses (Mottaran, et al., 2002).

Ethanol induced liver damage is often zonated within the liver. Notable, ethanol induced CYP 2E1 expression is mainly in the perivenous regions in the liver (Fang et al., 1998). In vitro studies using HepG2 cells transfected to express CYP 2E1, have shown signs of DNA fragmentation and apoptotic cell death which was not seen in controls. Inhibitors of ethanol oxidation via CYP 2E1 have also showed that there is depletion in the ethanol induced apoptosis (Wu and Cederbaum, 2004).

It has been previously demonstrated that oxidative stress plays a crucial role in the pathogenesis of ethanol induced liver damage. Ethanol induced oxidative stress is associated with the generation of ROS and RNI that leads to an imbalance in the pro-oxidant and anti-oxidant levels in the tissues (Arteel, 2003). Acetaldehyde, produced from the oxidative metabolite of ethanol leads to the peroxidative damage to membrane lipids leading to cellular damage. Moreover, acetaldehyde has the ability to interact with the cellular proteins such as enzymes, microsomal proteins and microtubules leading to the impairment of
normal protein functions (Rintala, *et al*., 2000), that has been proposed to play a key role in alcoholic liver diseases. Furthermore acetaldehyde can interact with DNA to form carcinogenic DNA adducts such as N²-ethyl-2'-deoxyguanosine (Brooks and Theruvathu, 2005). Numerous in-vitro and vivo studies had shown that acetaldehyde has a direct mutagenic and carcinogenic effect. It causes point mutation in human lymphocytes, induces sister chromatid exchanges and gross chromosomal aberrations (Obe *et al*., 1986, Dellarco 1988, Helender and Lindahl-Kidssling, 1991). Ethanol toxicity is associated with the induction of signalling cascade leading to the activation of transcription of NF-κB that results in the expression of inflammatory mediators including cytokines (TNF-α, IL-6, IL-12) (Iimuro *et al*., 1997) chemokines, lipid mediators, inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2). TNF-α further initiate the production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI’s) causing liver damage due to oxidative stress (Barnes and Karin, 1997; Nanji *et al*., 1999).

Now a day, there is growing interest in elucidating the role and mechanism of the phytochemicals as free radical scavengers and inhibitors of oxidative stress. In fact, the pharmacological effects of many traditional drugs have been ascribed to the presence of flavonoid compounds (Pietta, 2000), due to their ability to inhibit certain enzymes and their antioxidant activity.

This chapter has been sub divided into three studies as in the present chapter, three modulators viz, *Fagonia cretica*, Chrysin and Diosmin, have been used to ameliorate the ethanol induced hepatotoxicity in Wistar rats.

*Fagonia cretica* L., (previously known as *Fagonia arabica*) a tropical herb belonging to family Zygophyllaceae, commonly known as “Dhamasa”. It is a small spiny under shrub 1 to 3 feet height, mostly found in dry calcareous rocks throughout the Mediterranean region of Africa, Afghanistan, India and Pakistan (Rizvi *et al*., 1996; Chopra *et al*., 1982; Hooker 1881.). It is reputed medicinal plant in scientific and folkloric literature, and its medicinal values are well documented (Saeed, 1969). Different parts of this herb have been used to cure various ailments, including hematological, neurological, endocrinological and inflammatory disorders (Chopra *et al*., 1982; Saeed and Wahid, 2003). It has also been reported to contain wide variety of antioxidants and triterpenoids saponins (Miyase *et al*., 1996; Khalik *et al*., 2000). The plant is bitter in taste and is used for the treatment of fever, thirst, vomiting, dysentery, asthma, urinary discharges, liver trouble, typhoid, toothache, stomach troubles and skin diseases (Baquar, 1989). Its infusion is effective as a cooling agent in stomatitis. It is known to purify blood and also acts as a deobstruent (Said, 1996). It is also used for skin diseases,
small pox and for endothermic reaction in the body (Watt, 1972). The twigs of the plant are used as remedy for snake bite and also applied externally as paste on tumours and for the swellings of neck (Rizvi et al., 1996). In context with the use of *Fagonia cretica* as potent hepatoprotective agent in indigenous system of medicine, the present study was conducted to evaluate the hepatoprotective efficacy of *Fagonia cretica* against ethanol induced oxidative stress and liver damage at molecular level and to elucidate its role in modulating ethanol metabolizing enzymes.

Chrysin (5, 7-dihydroxyflavone), which is the focus of present study, is a flavone. The flavonoid, Chrysin is present at high levels in honey, propolis and many plant extracts (Siess et al., 1996; Williams et al., 1997). It has been shown to possess several beneficial pharmacological activities, such as an antioxidant (Lapidot et al., 2002), anti-hypertensive (Villar et al., 2002), anti-diabetogenic (Lukacinova et al., 2008), anti-inflammatory (Cho et al., 2004), anticancer (Habtemariam, 1997; Cardenas et al., 2006), anti-estrogenic (Machala et al., 2001), and anxiolytic activities (Wolfman et al., 1994). Chrysin has recently shown to be a potent inhibitor of aromatase (Sanderson et al., 2004) and of human immunodeficiency virus activation in models of latent infection (Critchfield et al., 1996). On the basis of these considerations, the present study was conducted to elucidate the influence of Chrysin on the ethanol metabolising enzymes and to evaluate hepatoprotective efficacy against ethanol mediated liver injury.

Diosmin (Diosmetin-7-O-rutinoside), a naturally occurring flavones glycoside readily obtained by dehydrogenation of hesperidin, found abundantly in the pericarp of various citrus (Campanero et al., 2010). Diosmin 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). Keeping in view the above biological activities of Diosmin, the present study was designed to made an attempt to evaluate the preventive efficacy of Diosmin against ethanol induced hepatic damage in Wistar rats by modulating ethanol metabolizing enzymes (CYP 2E1, ADH and Catalase), inflammatory cytokines (TNF-α, NF-κB, COX-2 and iNOS).
2. Treatment regimen

In this study, we have evaluated the preventive efficacy of *Fagonia cretica*, *chrysir* and *diosmin* against ethanol induced hepatic toxicity. Thirty female Wistar rats divided into five groups, each with six animals. Group I as control receive vehicle (Distilled water) only followed by Group II, III and IV were treated orally with ethanol (5, 8, 10 and 12g/kg b. wt per week) for 28 days. While groups III and IV were administered with modulator (*Fagonia cretica*/Chrysin/Disomin) orally at doses D1 and D2 respectively 1 hour prior to ethanol treatment. Group V was given only modulators (dose D2).

After 28 days of ethanol administration, rats were sacrificed by cervical dislocation under mild anesthesia and blood has been taken by cardiac puncture for various serological parameters. Liver samples were taken at the same time for various biochemical parameters. For control and ethanol treated groups same slides for histopathology were used in each set of experiment.

<table>
<thead>
<tr>
<th>Groups (n=6)</th>
<th>Treatment Regimen</th>
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<tbody>
<tr>
<td></td>
<td>1-7(^{th}) Day</td>
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<tr>
<td>Group I</td>
<td>Control</td>
</tr>
<tr>
<td>Group II</td>
<td>Toxicant</td>
</tr>
<tr>
<td>Group III</td>
<td>Ethanol 5g/kg b.wt + D1</td>
</tr>
<tr>
<td>Group IV</td>
<td>Ethanol 5g/kg b.wt + D2</td>
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<tr>
<td>Group V</td>
<td>Modulator High dose (D2)</td>
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</table>
3. Results

3.1. Study 1: Amelioration of ethanol induced liver toxicity by *Fagonia cretica*

3.1.1. Effect of administration of methanolic extract of *Fagonia cretica* on ethanol metabolizing enzymes

In ethanol treated groups, liver tissues showed significant (p<0.001) enhancement in ADH activities (111%) as compared with control group. Enhancement in liver ADH levels is restored by 80% (p<0.01) and 103% (p<0.001) at dose D1 and D2 in liver as compared with ethanol treated group. Whereas no changes in ADH activity were observed in only D2 groups as compared with the control group.

Ethanol caused significant induction of CYP 2E1 (p<0.001) in hepatic (80%) tissues as compared with control group. Treatment with *Fagonia cretica* brought back the level of CYP 2E1 to normal in hepatic (D1= 58%, p<0.01, D2= 82.94%, p<0.001) tissues.

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 tissue of ethanol treated group when compared with control. *Fagonia cretica* significantly restores the level of xanthine oxidase (XO) activity by 47% & 77% in liver tissue at dose D1 (p<0.01) and D2 (p<0.001) respectively. Only D2 group showed no significant change as compared to control group.

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

MDA formation was measured to demonstrate the oxidative damage in ethanol induced liver injury of Wistar rats. A significant (p<0.001) amplification of the MDA formation was found in the ethanol treated group in hepatic 84% tissue when compared with controls. 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 by 50% and 84% respectively when compared to ethanol treated group. *Fagonia cretica* alone did not show any significant difference as compared to control.

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

Ethanol treatment was found to diminish hepatic antioxidants GSH (55%) GPx (53%), GR (48%) and Catalase (58%) as compared to corresponding control group (p<0.001). Treatment of *Fagonia cretica* significantly increases the level of GSH, GPX, GR and Catalase...
in liver at dose D1 and D2 by 25 & 35%, 27 & 39%, 31 & 46% and 37 & 53% respectively. Which indicates antioxidant property of *Fagonia cretica* against ethanol induced oxidative stress.

### 3.1.5. *Fagonia cretica* treatment attenuates ethanol induced hepatotoxicity

Ethanol treated groups showed 56, 84 & 44% increase in serum AST, ALT and LDH levels respectively. Administration with *Fagonia cretica* was found effective (p<0.01, NS, p<0.01) in the normalization in these serum toxicity markers by 38, 45 and 27% at D1 and 52, 83 and 43% at D2 when compared to ethanol treated group (Table 1).

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

TNF-α levels in the hepatic tissue of the ethanol treated rats was significantly elevated (p<0.001) as compared with control group. *Fagonia cretica* administration showed a significant reduction in the hepatic TNF-α levels at both doses D1 (p<0.01) and D2 (p<0.01) as compared with the ethanol treated group, whereas no significant change in the TNF-α levels were found in only D2 group as compared to control group (Figure 1).

### 3.1.7. Histopathology

Analysis of tissue sections of animals from different treatment groups under microscope (∗10 and ∗40 enlargement) revealed marked changes when compared with control group animals (Fig. 2A). In the ethanol-treated animals, there was an apparent inflammatory response around the central vein in terms of infiltration of inflammatory cells. Moreover, ethanol also caused vacuolar degeneration and pronounced necrosis around the central vein (Fig. 2B) in liver tissue. In contrast, *Fagonia cretica* extract administration at both the doses (100 and 200 mg/kg b. wt) protected the liver histology against ethanol-induced alterations (Fig. 2C and D). Only *Fagonia cretica* administration (200 mg/kg b. wt) does not show any alterations from normal liver histology (Figure 2E).
Table 1: Effect of *Fagonia cretica* administration on ethanol mediated alteration in serum enzyme activities of liver toxicity.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ethanol</th>
<th><em>F. cretica</em> D1 + Ethanol</th>
<th><em>F. cretica</em> D2 + Ethanol</th>
<th>Only <em>F. cretica</em> D2</th>
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<tr>
<td><strong>Aspartate aminotransferase</strong> (AST or SGOT) (IU/L)</td>
<td>47.39 ± 3.44</td>
<td>73.89 ± 3.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55.74 ± 3.1&lt;sup&gt;**&lt;/sup&gt;</td>
<td>49.44 ± 3.9&lt;sup&gt;***&lt;/sup&gt;</td>
<td>47.05 ± 3.62</td>
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<tr>
<td><strong>Alanine aminotransferase</strong> (ALT or SGPT) (IU/L)</td>
<td>11.54 ± 1.39</td>
<td>21.21 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.68 ± 1.6&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>11.59 ± 1.5&lt;sup&gt;**&lt;/sup&gt;</td>
<td>11.07 ± 1.11</td>
</tr>
<tr>
<td><strong>Lactate dehydrogenase</strong> (LDH) (nmol NADH oxidised/min/mg protein)</td>
<td>194.4 ± 8.33</td>
<td>280.4 ± 8.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>228.9 ± 11.1&lt;sup&gt;**&lt;/sup&gt;</td>
<td>196 ± 10.5&lt;sup&gt;***&lt;/sup&gt;</td>
<td>194.2 ± 9.08</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.

<sup>*P</sup> <0.05, <sup>**P</sup> <0.01 and <sup>***P</sup> <0.001 compared with the corresponding value for ethanol treated group.

*Fagonia cretica* low dose (100mg/kg b. wt) and F.C (D2): *Fagonia cretica* high dose (200mg/kg b. wt)
Figure 1: Effect of *Fagonia cretica* administration on TNF-α expression

Representative graph showed the effect of *Fagonia cretica* treatment on TNF-α level. Each value represents mean ± S.E., n = 6

F.C (D1): *Fagonia cretica* low dose (100mg/kg b. wt) and F.C (D2): *Fagonia cretica* high dose (200mg/kg b. wt)

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

**P <0.01 and ***P <0.001 compared with the corresponding value for ethanol treated group.
Figure 2: Histopathology of liver tissue of rats treated with different treatments in this study.

Representative photomicrograph showed the effect of *Fagonia cretica* treatment on ethanol induced histopathological changes in liver tissue. **2A)** Liver section of rat control group showing structural intactness and normal architecture. **2B)** Ethanol treated group (400x). **2C)** Co-administration of *Fagonia cretica* (at a dose of 100mg/kg b.wt) and ethanol. (400x). **2D)** Co-administration of *Fagonia cretica* (Dose 200mg/kg b Wt) and ethanol (400x). **2E)** Slide showing liver histology of rats treated with only *Fagonia cretica* at a dose of 200mg/kg b wt. (400x).
3.2. Study 2: Amelioration of ethanol induced liver toxicity by Chrysin

3.2.1. Effect of chrysin on body weight and relative organ weight

Figure 3 shows per week average body weight of the animals treated with different treatment regimes for 4 weeks. After 4 weeks of study, rats treated with ethanol only showed a slight decrease in body weights (0 day= 202 ± 1.07; 28th day = 200.8 ± 2.1). However, body weight of rats of group III (0 day= 190 ± 2.3; 28th day= 198 ± 2.08) and group IV (0 day= 186 ± 2.8; 28th day= 194.4 ± 2.18) indicates 8 grams increase in body weights in both groups, this elevation in body weight is almost similar to the increase in body weight of control animals (0 day= 202 ± 2.6; 28th day= 211.2 ± 2.8).

Figure 4, shows the relative liver weights of animals. Slight decrease in relative liver weights have been observed in the rats treated with ethanol only (Liver = 2.15 ± 0.05) as compared with control group (Liver= 2.41 ± 0.09). This slight decrease in relative organ weight has been restored by the chrysin administration (Liver: D1= 2.37 ± 0.17; D2= 2.41 ± 0.11). However there is no significant change in the relative organ weights in only chrysin treated group.

3.2.2. Effect of chrysin on ethanol metabolizing enzymes

Ethanol caused significant induction of CYP 2E1 (p<0.001) in hepatic (83%) tissues as compared with control group. Treatment with chrysin brought back the level of CYP 2E1 to normal in hepatic (D1= 41%, p<0.01, D2= 70%, p<0.001) (Figure 5).

In ethanol treated groups, (Figure 6) liver showed significant (p<0.001) enhancement in ADH activities (35%) as compared with control group. Enhancement in liver ADH levels is restored by 12% & 24% at dose D1 and D2 in liver tissues as compared with ethanol treated group, whereas no changes in ADH activity were observed in only D2 groups.

3.2.3. Effect of chrysin on hepatic membrane damage (Lipid Peroxidation)

MDA formation was measured to demonstrate the oxidative damage in ethanol induced liver injury of Wistar rats. A significant (p<0.001) amplification of the MDA formation was found in the ethanol treated group in hepatic (157%) tissue when compared with controls.

We have observed that treatment with chrysin at D1 and D2 leads to the significant restoration (p<0.01 and p<0.001 respectively) of membrane integrity in liver (D1= 62%; D2= 125%) when compared to ethanol treated group (Figure 7). Chrysin alone did not show any significant difference as compared to control.

3.2.4. Effect of chrysin on Xanthine oxidase activity
Xanthine oxidase (XO) reflected significant increase (p<0.001) in the enzyme activity in hepatic tissue of ethanol treated group when compared with control. Chrysin significantly restores the level of xanthine oxidase (XO) activity by 66 & 92% in liver tissue at dose D1 and D2 respectively. Only D2 group showed no significant change as compared to control group.

3.2.5. Chrysin treatment restores the activities of hepatic antioxidants

Ethanol treatment was found to diminish hepatic antioxidants GSH (50%) GPx (37%), GR (40%) and Catalase (34%) as compared to corresponding control group (p<0.001). Treatment of chrysin significantly increases the level of GSH, GPX, GR and Catalase in liver at dose D1 and D2 by 16 & 38%, 19 & 21%, 7 & 22%, 10 & 18% respectively. Which indicates antioxidant property of chrysin against ethanol induced oxidative stress.

3.2.6. Chrysin attenuates ethanol induced hepatotoxicity

Ethanol treated group showed 112% (p<0.001), 98% (p<0.001) and 45% (p<0.001) increase in serum AST, ALT and LDH levels respectively as compared with the control group. Chrysin administration was found significantly effective in the normalization in these serum toxicity markers by 38% (p<0.001), 45% (p<0.001) and 27% (p<0.01) at D1 and 67% (p<0.001), 67% (p<0.001) and 43% (p<0.001) at D2 when compared to ethanol treated group (Figures 8 & 9).

3.2.7. Effect of chrysin treatment on hepatic TNF-α level

TNF-α levels in the hepatic tissue of the ethanol treated rats was significantly elevated by 32% (p<0.001) as compared with control group. Chrysin administration showed a significant reduction by 22% and 29% in the hepatic TNF-α levels at both doses D1 (p<0.01) and D2 (p<0.01) respectively as compared with the ethanol treated group, whereas no significant change in the TNF-α levels were found in only D2 group as compared to control group (Figure 10).

3.2.8. Effect of chrysin administration on caspase 3, 7 and 9 activities

Figure 11 shows the expression of caspase 3, 7 and 9 in liver tissue of rats. Ethanol treated group shows a significant elevation of caspase 3, 7 and 9 by 225%, 210% and 185% respectively as compared to control group. This elevation in caspase activities is significantly depleted by chrysin administration. Caspase 3 activity was depleted by 61% (NS) and 176% (p<0.05), caspase 7 activity by 95% (p<0.05) and 158% (p<0.01) and caspase 9 by 97%
(p<0.05) and 158% (p<0.01) at doses D1 and D2 respectively, as compared with only ethanol treated group. Animals treated with only high dose of chrysin did not show any significant change as compared to control group.

3.2.9. **Histopathology of liver tissue**

Analysis of tissue sections of animals from different treatment groups under microscope (Figure 12 and 400x enlargement) revealed marked changes when compared with control group animals (Figure 12A & 12B). In the ethanol treated animals there was an apparent inflammatory response around central vein in terms of infiltration of inflammatory cells. Moreover ethanol also caused vacuolar degeneration and pronounced necrosis around central vein (Figure 12 C) in liver tissue. In contrast chrysin administration at both the doses (20 and 40 mg/kg b.wt.) protected the liver histology against ethanol induced alterations (Figure 12D & 12E). Only chrysin administration (40 mg/kg b.wt.) does not show any alterations from normal liver histology (Figure 12F).
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Amelioration of ethanol induced liver damage...

Figure 3: Effect of chrysin administration on average increase in body weight per week of rats

![Graph showing effect of chrysin administration on average increase in body weight per week of rats.](image)

Figure 4: Effect of chrysin treatment on relative liver weight of rats

![Graph showing effect of chrysin treatment on relative liver weight of rats.](image)

Representative graph showed the relative liver weight of rats at the end of the study.
Figure 5: Effect of chrysin on CYP 2E1 activity

Representative graph shows ethanol induced CYP 2E1 activity and its restoration by chrysin administration.
Each value represents mean ± S.E., n = 6
# P < 0.001 as compared with the corresponding value for control group.
**P < 0.01 and ***P < 0.001 compared with the corresponding value for ethanol treated group.
Figure 6: Effect of chrysin treatment on ADH activity in Liver tissues

Each value represents mean ± S.E., n = 6
++p<0.01 as compared with the corresponding value for control group.
NS: Non-significant; *P < 0.05 compared with the corresponding value for ethanol treated group.
Figure 7: Inhibition of Lipid peroxidation by chrysin treatment

\[
\begin{array}{cccccc}
\text{nmol MDA formed/hr/g tissue} \\
\text{Control} & \text{Ethanol} & \text{Chrysin 20mg/kg + Ethanol} & \text{Chrysin 40mg/kg + Ethanol} & \text{Only Chrysin 40mg/kg} \\
\end{array}
\]

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 ethanol treated group.

Figure 8: Effect of chrysin administration on serum markers of liver toxicity AST and ALT

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 ethanol treated group.
Figure 9: Effect on cytotoxicity marker lactate dehydrogenase (LDH) by chrysin administration

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 ethanol treated group.

Figure 10: Effect of chrysin treatment on hepatic TNF-α level

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 ethanol treated group.
Chrysin D1: 20mg/kg b. wt and Chrysin D2: 40mg/kg b. wt.
Figure 11: Effect of chrysin administration on caspase 3, 7 and 9 activities

![Caspase activity graph]

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 ethanol treated group.

Chrysin D1: 20mg/kg b. wt and Chrysin D2: 40mg/kg b. wt.
Figure 12: Effect of chrysin administration on ethanol alterations in liver histopathology

**Histopathology of liver tissue:** A (100x) & B (400x): Control group. C) Ethanol treated group (400x), D) Chrysin (20mg/kg b.wt) + ethanol group (400x), E) Co-administration of Chrysin (Dose 40mg/kg b Wt) showing almost normal architecture (400x). F) Only chrysin D2 group
3.3. Study 3: Attenuation of ethanol induced liver toxicity by Diosmin

3.3.1. Effect of Diosmin on body weight and relative liver weight of rats

Figures 13 and 14 shows respective body weight and relative organ weight of rats treated with Diosmin (10 and 20mg/kg b. wt) for 4 weeks. Our results demonstrate that there was a slight decrease in the body weight (per week) and liver body weight ratio of rats that ingested alcohol only compared to normal group. Diosmin administration regains body weight and liver body weight ratio to normal. This indicates that administration with Diosmin decreased weight loss due to chronic alcohol ingestion.

3.3.2. Effect of Diosmin on ethanol metabolizing enzymes

Ethanol caused significant induction of CYP 2E1 (p<0.001) in hepatic (77.82%) tissue as compared with control group. Treatment with diosmin brought back the level of CYP 2E1 to normal in hepatic (D1= 44.79%, p<0.01, D2= 68.33%, p<0.001) tissue. (Figure 15A).

In ethanol treated groups, Figure 15B, liver showed significant (p<0.01) enhancement in ADH activities (32%) as compared with control group. Enhancement in liver ADH levels is restored by 7% and 31.35% at dose D1 (NS) and D2 (p<0.05) in liver as compared with ethanol treated group, whereas no changes in ADH activity were observed in only D2 groups.

3.3.3. Effect of diosmin on Xanthine oxidase activity

Xanthine oxidase (XO) reflected significant increase (82.45%, p<0.001) in the enzyme activity in hepatic tissue of ethanol treated group when compared with control. Diosmin significantly restores the level of Xanthine oxidase (XO) activity by 56.14% (p<0.01) & 77.19% (p<0.001) in liver tissue at dose D1 and D2 respectively. Only D2 group showed no significant change as compared to control group.

3.3.4. Diosmin treatment restores the activities of hepatic antioxidants

Ethanol treatment was found to diminish hepatic antioxidants GSH (56.25%) GPx (47.11 %), GR (48.83 %) and Catalase (36.72%) as compared to corresponding control group (p<0.001). Treatment of diosmin significantly increases the level of GSH, GPX, GR and Catalase in liver at dose D1 and D2 by 17.18% (NS) & 25% (p<0.01), 37.3% (p<0.01) & 49.86% (p<0.001), 21.63% (p<0.01) & 44.90% (p<0.001) and 32.65% (p<0.01) & 40.51% (p<0.001) respectively. Which indicates antioxidant property of diosmin against ethanol induced oxidative stress.
3.3.5. **Diosmin attenuates ethanol induced hepatotoxicity**

Ethanol treated groups showed 116.91, 102.03 and 45.2% increase in serum AST, ALT (Figure 16A) and LDH levels (Figure 16B) respectively as compared with the control group. Administration with diosmin was found significantly (p<0.01, p<0.001, p<0.001) effective in the normalization in these serum toxicity markers by 44.18, 33.35 and 15.58% at D1 and 97.7, 81.1 and 38.32% at D2 respectively when compared to ethanol treated group.

3.3.6. **Effect of diosmin on hepatic membrane damage (Lipid Peroxidation)**

MDA formation was measured to demonstrate the oxidative damage in ethanol induced liver injury of Wistar rats. A significant (p<0.001) amplification (177.1%) of the MDA formation was found in the ethanol treated group in hepatic tissue when compared with control. We have observed that treatment with diosmin at D1 and D2 leads to the significant restoration (p<0.01 and p<0.001 respectively) of membrane integrity in liver by 90.77 and 137.55% when compared to ethanol treated group (Figure 17). Diosmin alone did not show any significant difference as compared to control.

3.3.7. **Restoration of TNF-α level by diosmin.**

Ethanol treated groups showed significant (P<0.001) elevation in hepatic TNF-α levels (307.2 ± 12.89; 28.05%) as compared with the control group (239.9 ± 8.6), while diosmin administration at dose D1 (P<0.01) and D2 (p<0.001) restores the elevated levels of TNF-α by 21.30% (256.1 ± 10.89) and 27.63% (240.9 ± 9.15) respectively as compared with the ethanol treated group (Figure 18).

3.3.8. **Expression of NF-κB, COX-2 and iNOS**

Hepatic expressions of NF-κB, COX-2 and iNOS have been shown in the figures 19I, 19II and 19III respectively. In the ethanol treated group, there was higher number of cells showing expression of these proteins as indicated by the brown stains. Expression of these proteins (NF-κB, COX-2 and iNOS) is markedly suppressed in the diosmin treated groups. For immunohistochemical analysis, brown colour indicates specific immune-staining of these proteins and light blue colour indicates haematoxylin staining (original magnification: 400x).
3.3.9. 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 (Figure 20A) animals (Figure 20). In the ethanol-treated animals, there was an evident vacuolar degeneration and pronounced necrosis around the central vein. Moreover, ethanol causes apparent inflammatory response around the central vein in terms of infiltration of inflammatory cells (Figure 20B) in liver tissue. In contrast, diosmin administration at both the doses (10 and 20 mg/kg b. wt) protected the liver histology against ethanol-induced alterations (Figure 20C and 20D). Only diosmin administration (20 mg/kg b. wt) does not show any alterations from normal liver histology (Figure 20E).
Figure 13: Effect of Diosmin on body weight of rats

![Graph showing average increase in body weight (g) per week.]

- **Control**
- **Ethanol**
- **Diosmin D1**
- **Diosmin D2**
- **Only Diosmin D2**

Figure 14: Effect of Diosmin on relative liver weight of rats

![Graph showing relative liver weight.]

- **Control**
- **Ethanol**
- **Diosmin D1**
- **Diosmin D2**
- **Only Diosmin D2**
Figure 15: Effect of Diosmin on ethanol metabolizing enzymes, CYP 2E1 (15A) and ADH (15B)

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 ethanol treated group.
Diosmin D1: 10mg/kg b. wt and Diosmin D2: 20mg/kg b. wt.
Each value represents mean ± S.E., n = 6
++P < 0.01 compared with the corresponding value for control group.
NS: Non-significant, *P<0.05, *P <0.01 and ***P <0.001 compared with the corresponding value for ethanol treated group.
Diosmin D1: 10mg/kg b. wt and Diosmin D2: 20mg/kg b. wt.
Figure 16: Diosmin attenuates ethanol induced hepatotoxicity AST and ALT (16A) and LDH (16B)

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 ethanol treated group.

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

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

(16B)
Figure 17: Effect of diosmin on hepatic membrane damage (Lipid Peroxidation)

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 ethanol treated group.
Diosmin D1: 10mg/kg b. wt and Diosmin D2: 20mg/kg b. wt.
Figure 18: Restoration of TNF-α level by diosmin

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 ethanol treated group.

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

Amelioration of ethanol induced liver damage...

Figure 19: Effect of diosmin administration on the expression of (19I) NF-κB, (19II) COX-2 and (19III) iNOS.

(19I): Photomicrograph represents the effect of Diosmin treatment on ethanol induced NF-κB expressions in rat liver. Brown color indicates specific immunostaining of NF-κB, and light colour indicates haematoxylin staining. (19A): Liver sections of control rats. (19B): showing hepatic sections of ethanol fed rats showing higher expression of NF-κB (arrows). (19C & D): Liver sections of diosmin treated groups (D1 and D2 respectively). (19E): Only diosmin D2 treated group.

Diosmin D1: 10mg/kg b. wt and Diosmin D2: 20mg/kg b. wt.
**19II**: Immunohistochemistry of COX-2 in liver. Positively stained COX-2 staining yielded a brown-coloured product (arrows). Vehicle treated control rat, 400x (A). Ethanol treated, 400x (B); Diosmin 10mg/kg b. wt plus ethanol treated, 400x (C); Diosmin (20mg/kg b wt) plus ethanol treated, 400x (D); Only diosmin (20mg/kg b wt) treated, 400x (E).
**Amelioration of ethanol induced liver damage**

*(19III):* Immunohistochemistry of iNOS in the liver of diosmin treated rats. Positively stained iNOS staining yielded a brown-coloured product (arrows). Vehicle treated control rat, X400 (A). Ethanol treated, X400 (B); Diosmin 10mg/kg b. wt plus ethanol treated, X400 (C); Diosmin (20mg/kg b wt) plus ethanol treated, X400 (D); Only diosmin (20mg/kg b wt) treated, X400 (E).
Figure 20: Effect of diosmin on histopathology of the liver tissue

A) Photomicrographs showed the liver sections of rats of control group showing structural intactness and normal architecture. B) Ethanol treated group showing inflammatory response around central vein (arrow) with vacuolar degeneration and necrosis (400x). C) Co-administration of diosmin (at a dose of 10mg/kg b.wt) with ethanol group showing lesser vacuolar degeneration and lesser inflammatory response around central vein (400x). D) Co-administration of diosmin (Dose 20mg/kg b Wt) showing almost normal architecture (400x). E) Slide showing liver histology of rats treated with only diosmin at a dose of 20mg/kg b wt. (400x).
4. Discussion

The consequences of alcohol misuse are life-threatening in many countries. It has been estimated that more than 200 million people throughout the world suffer from alcohol dependence (WHO report 2008). Alcohol consumption has been related to several alcohol related illnesses including cancer, liver pathology, myopathy, cerebellar atrophy, testicular injury and immune-suppression (Ishii et al., 1997; Guidot and Roman, 2002). In the present study an attempt has been made to prevent ethanol induced liver toxicity by suppressing ethanol metabolism enzymes, inhibiting ethanol induced oxidative stress, preventing ethanol induced inflammation and cell death (apoptosis) by natural products (*Fagonia cretica*, chrysin and diosmin). Per week sequential increased dose of ethanol from 5g/kg b. wt to 12g/kg b. wt was used to induce maximum tissue damage and to overcome the tolerance produced by the ethanol consumption at same dose.

It has been reported that, chronic ethanol consumption is associated with the reduction in the adipose tissue content in the body that results in the reduction of body weight gain (Aruna et al., 2005; Das and Vasudevan, 2005). Contrarily, administration of chrysin and diosmin modulates alteration in body weight gain and relative liver weight of rats treated with ethanol.

Many pathways have been suggested as playing a key role in ethanol induced oxidative stress (Arteel, 2003; Tsukamoto and Lu, 2001) Some of these include redox state changes; production of the reactive product acetaldehyde; damage to mitochondria; direct or membrane effects caused by hydrophobic ethanol; ethanol-induced hypoxia; ethanol effects on the immune system and altered cytokine production; ethanol induction of CYP2E1; ethanol mobilization of iron; effects on antioxidant enzymes and chemicals, particularly mitochondrial and cytosolic glutathione; 1-electron oxidation of ethanol to the 1-hydroxyl ethyl radical (Nordmann, et al., 1992; Bondy, 1992).

Ethanol metabolism by alcohol dehydrogenase increases NADH resulting in the induction of xanthine oxidase activity with subsequent production of aggressive oxygen species. Increased generation of oxygen and ethanol derived free radicals occurs at the microsomal level, especially during the action of the ethanol inducible CYP2E1 (MEOS). CYP2E1 has been shown to be able to produce superoxide anion, hydrogen peroxide and ethanol derived hydroxyethyl free radical. MEOS aggravates oxidative stress directly as well as indirectly by impairing the defence system against it. (Kalousova et al., 2004; Koch et al., 2004; Sun et al., 2001; Zima et al., 1993; Zima et al., 2001). Our results are also in complete conformity with the above findings, that ethanol administration to rats results in the elevation of xanthine.
oxidase and ADH activities with subsequent induction of CYP 2E1 in the liver tissue, whereas treatment with *Fagonia cretica*, chrysin and diosmin restores the activities of ethanol metabolising enzymes (ADH and CYP 2E1) and xanthine oxidase in liver tissue of rats. This restoration in these enzymes could predict that administration with these modulators could prevent against the deleterious effects of ethanol induced free radical damage to liver.

Ethanol induced free radical generation was associated with enhanced lipid peroxidation, protein carbonyl formation, formation of the 1-hydroxyl ethyl radical, formation of lipid radicals, and decreases in hepatic antioxidant defence (Catalase, glutathione peroxidase, GST and especially GSH) (Rouach et al., 1997; Polavarapu et al., 1998; Knecht et al., 1995; Nanji et al., 1994). Glutathione, an important cellular reductant protein involved in protection against free radicals, peroxides and other toxic compounds (Gerster, 1995). Glutathione peroxidase has a well established role in protecting cells against oxidative stress and catalase, which acts as preventative antioxidant, play an important role in protection against the deleterious effects of lipid peroxidation associated with ethanol intoxication (Dinkova-kostova and Talalay, 1999).

Generation of ROS, from acetaldehyde oxidation during ethanol metabolism may contribute to the oxidative stress in liver tissues (Rodrigo et al., 1998 and Fernandez-Checha et al., 1987) which is evident from significant decrease in the activities of Catalase, Glutathione and dependent enzymes GPx and GR, in ethanol treated rats. The decreased hepatic GSH in ethanol intoxicated rats could be the result of hexose monophosphate (HMP) shunt impairment and thereby altering oxidant: antioxidant status in the cells (Lu, 1999). During ethanol ingestion the activities of some intracellular antioxidants have been reported to decrease with the increase in lipid peroxidation levels (Diplock et al., 1994) and this fact is concomitant with the results of the present study, which was also in agreement with reports of Fernandez and Videla (1981) and Jaya et al., (1993). The analysis of antioxidant status in our study indicates that both non-enzymatic and enzymatic antioxidants were decreased due to ethanol administration. Administration of *Fagonia cretica*, chrysin and diosmin with alcohol significantly modulates the anti-oxidant status in the liver of rats, suggesting the modulating effect of chrysin on cellular antioxidant defence.

Cellular damage exhibits good correlation with the enzyme leakage (Sherawat and Sultana, 2006). Serum AST, ALT and LDH are the most sensitive markers employed in the diagnosis of hepatic damage (Sallie et al., 1991). The present study entirely collaborates with the above findings that chronic ethanol administration leads to elevated levels of serum toxicity markers of liver (ALT, AST and LDH), that are indices of hepatic dysfunction. The increase
in the activities of these enzymes in serum and subsequent fall in the tissue might be due to the leakage of these cytosolic enzymes into the circulatory system resulting from free radical induced liver damage during ethanol administration. This is indicative of the onset of hepato-cellular damage due to liver dysfunction and disturbance in the biosynthesis of these enzymes, with alteration in the membrane permeability. Administration with *Fagonia cretica*, chrysin and diosmin to rats prevented ethanol-induced hepatotoxicity, as indicated by a precipitous drop in serum ALT, AST, and LDH activity, possibly by maintaining the hepatocellular membrane integrity. This is an indicator of possible hepatoprotective efficacy offered by these modulators compared with the ethanol intoxicated animals.

Kupffer cells, the resident macrophage in the liver, are critical to the progression of ethanol induced liver injury, in part, because of their role as an important producer of TNF-α (Yin et al., 1999). Numerous reports have demonstrated that TNF-α plays a pivotal role in the ethanol induced liver pathology (Honchel et al., 1992; Ji et al., 2004). Activation of transcription factor NF-κB by TNF-α is one of the myriad actions of TNF-α that causes genes to generate potentially cell damaging oxidative enzymes such as NADP oxidase, cyclooxygenase (COX-2) and iNOS as well as further release of TNF-α and other pro-inflammatory cytokines (Nanji et al., 2003). In the present strategy of inducing hepatic injury by chronic ethanol administration, our results are in complete accordance with the above reports that the levels of necrosis and inflammatory markers (TNF-α, NF-κB, iNOS and COX-2) increased in the rats treated with ethanol only. Furthermore, *Fagonia cretica*, chrysin and diosmin administrations in the present study have been found to restore these elevated levels of inflammatory cytokines.

ROS production is detrimental when excessive amounts are produced leading to tissue damage. Ethanol exposure causes a shift in oxidant/antioxidant homeostasis leading to oxidative stress (Arteel, 2003). ROS can act as a second messenger and induce apoptosis, in part, by signalling through the MAPK pathway (Chen et al., 1995; Lu et al., 2007). Oxidative stress and lipid peroxidation products generated during ethanol toxicity induce apoptosis in liver cells by increasing the expression of apoptotic regulatory proteins and adaptors, such as Fas ligand and FasL receptor, caspase-2, caspase-3, caspase-7, caspase-9 and Bax (de Villiers et al., 2007). In the present study we have also observed that ethanol administration to rats leads to the elevation in caspase activities confirming the induction of cell death or apoptosis of liver cells and we have also observed that treatment with *Fagonia cretica*, chrysin and diosmin significantly normalizes this elevation of caspase activities indicating preventive efficacy of these modulators against ethanol induced cell death.
It is clear from the results that ethanol induced hepatic damage can be characterised by the induction of CYP 2E1, activation of resident hepatic macrophages, ROS production, inflammatory cytokine expression and apoptosis. It is also confirmed from our results that treatment with natural antioxidant (Fagonia cretica, chrysin and diosmin) leads to the amelioration of the ethanol induced liver damage significantly.

In conclusion, in the present study, several mechanisms by which ethanol lead to hepatotoxicity were evaluated. Among them, generation of free radicals, imbalance in redox state, damage to mitochondria, peroxidation of membrane lipids and induction of TNF-α and activation of the NF-κB and its translocation to nucleus are the major mechanism revealing ethanol inducing liver toxicity. All these mechanisms lead to cell death and require ethanol metabolism to acetaldehyde. Findings from the present studies permits us to conclude that Fagonia cretica, chrysin and diosmin alleviates alcoholic liver injury via modulating ethanol metabolising pathway, inhibition of oxidative stress and repression of inflammation. This may represent a novel protective strategy against ethanol induced liver diseases.