Protective effects of silymarin, a milk thistle (Silybum marianum) derivative on ethanol-induced oxidative stress in liver

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The production of reactive oxygen species (ROS) is considered to be a major factor in oxidative cell injury. The antioxidant activity or the inhibition of the generation of free radicals is important in providing protection against such hepatic damage. Silymarin, derived from the milk thistle plant, Silybum marianum, has been used in traditional medicine as a remedy for diseases of the liver and biliary tract. In the present study, the effect of hepatoprotective drug silymarin on body weight and biochemical parameters, particularly, antioxidant status of ethanol-exposed rats was studied and its efficacy was compared with the potent antioxidant, ascorbic acid as well as capacity of hepatic regeneration during abstention. Ethanol, at a dose of 1.6 g/kg body wt/day for 4 wks affected body weight in 16-18 week-old male albino rats (Wistar strain weighing 200-220 g). Thiobarbituric acid reactive substance (TBARS) level, superoxide dismutase (SOD), and glutathione-s-transferase (GST) activities were significantly increased, whereas GSH content, and catalase, glutathione reductase (GR) and GPx (glutathione peroxidase) activities significantly reduced, on ethanol exposure. These changes were reversed by silybin and ascorbic acid treatment. It was also observed that abstinence from ethanol might help in hepatic regeneration. Silybin showed a significant hepatoprotective activity, but activity was less than that of ascorbic acid. Furthermore, preventive measures were more effective than curative treatment.

Keywords: Silybum marianum, Ascorbic acid, Ethanol, Oxidative stress, Silybin, Silymarin, antioxidant status

Alcohol has been implicated in the genesis of liver disease. Both its consumption and metabolism promote the production of inflammatory mediators that result in hepatotoxicity and fibrogenesis. With time, this leads to progressively severe liver injury and, eventually, causes cirrhosis. Toxic substances generated during the metabolism of alcohol in the liver may contribute to the development of alcoholic liver disease (ALD). These substances include highly reactive molecules that can damage vital cell components through oxidation. Oxidative stress is well recognized to be a key step in the pathogenesis of ethanol-associated liver injury.

Antioxidants such as certain enzymes, vitamins, and other substances protect cells against oxidation and an imbalance between oxidants and antioxidants can lead to oxidative stress, characterized by escalating cell damage. Oxidative stress and associated cellular injury promote inflammation. Antioxidants could have beneficial effects in reducing the incidence of ethanol-induced changes in cellular lipids, proteins and nucleic acids. They could act by reducing free radical production (e.g. chelators of redox-active iron derivatives), trapping free radicals themselves, interrupting the peroxidation process or reinforcing the natural antioxidant defence.

Silymarin, a mixture of three structural components: silibinin, silydianine and silychristine is extracted from the seeds and fruit of milk thistle, Silybum marianum (Compositae). These compounds have been shown to protect different organs and cells against a number of insults. In the present study, the effect of hepatoprotective drug silymarin on body weight and biochemical parameters, particularly, antioxidant status of ethanol-exposed rats was studied and its efficacy was compared with the potent antioxidant ascorbic acid, as well as capacity of hepatic regeneration during abstention. Efficacies of these experimental drugs were tested in animals either by simultaneous supplementation with ethanol (preventive treatment) or in the follow-up treatment after completion of ethanol administration (curative treatment).
Materials and Methods
Ethanol was purchased from Bengal Chemicals, Kolkata. Fine chemicals were from Sisco Research Laboratory (SRL), India, and Sigma Chemical Co., St. Louis, USA; and analytical grade chemicals from E. Merck or SRL. Silymarin was used in the form of silybin. Ascorbic acid and silybin were freshly dissolved in distilled water during treatment. Ethanol was diluted with distilled water to get desired concentration and fed orally, when necessary.

Animals and treatment
The male albino rats (16-18 weeks old) of Wistar strain weighing 200-220 g were housed in plastic cages inside a well-ventilated room, with the room temperature maintained at 25±2°C, with a 12-h light/dark cycle. Animals had free access of standard diet as recommended by the Veterinary and Animal Husbandry Department of Kerala Agriculture University; with some modifications. The diet contained 31% Bengal gram, 30% gilingelly oil cake, 28% wheat, 10% polished rice, 0.5% salt mixture, 0.3% vitamin-mineral mixture, and 0.2% yeast with fish or liver oil. Food and water were given ad libitum. Animals were weighed daily and their general condition and behaviour were recorded, including their daily intake of liquid.

The rats were divided into the following seven groups of 6 animals each. Group I: control - were fed normal diet and water; Group II: ethanol-treated (1.6 g ethanol/kg body wt/day for 4 wks); Group III: silybin + ethanol-treated (1.6 g ethanol and 200 mg silybin/kg body wt/day for 4 wks); Group IV: ethanol (1.6 g ethanol/kg body wt/day for 4 wks) followed by silybin treated (200 mg silybin/kg body wt/day for 4 wks); Group V: ascorbic acid + ethanol-treated (1.6 g ethanol and 1 g ascorbic acid/kg body wt/day for 4 wks); Group VI: ethanol (1.6 g ethanol/kg body wt/day for 4 wks) followed by ascorbic acid-treated (1 g ascorbic acid/kg body wt/day for 4 wks); and Group VII: ethanol treatment (1.6 g ethanol/kg body wt/day) for 4 wks and followed by 4 wks abstination.

At the end of the experimental period, the animals were sacrificed after overnight fast, by intraperitoneal injection of sodium pentobarbital (50 mg/kg body wt). The liver was dissected out and cleaned with ice-cold saline, blotted dry, and immediately transferred to the ice chamber for preservation. Various oxidative stress related non-enzymes such as ascorbic acid, thiobarbituric acid reactive substances (TBARS), glutathione (GSH); and enzymes such as catalase, superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione s-transferase (GST) were estimated. Animals of group II were sacrificed at the end of 4 wks of exposure to ethanol; and all other animals were sacrificed at the end of 8 wks of treatment period as described above. The Animal Ethics Committee of the Institution approved the procedures in accordance with the CPCSEA guideline.

Biochemical methods
Liver was homogenized in 0.25 M sucrose solution, and the whole homogenate was then diluted with 0.9% saline, finally diluted samples were used for the estimation of tissue protein10. Ascorbic acid content was estimated using thiourea reagent and 2,4-dinitrophenylhydrazine11. Lipid peroxidation (LPO) was estimated using trichloroacetic acid (TCA)-thiobarbituric acid (TBA)-HCl at 535 nm12. The reduced glutathione (GSH) content of the tissue was measured using DTNB reagent [5,5′-dithiobis(2-nitrobenzoic acid)]13. Catalase (EC 1.11.1.6) activity was measured by monitoring H2O2 decomposition at 240 nm14. The superoxide dismutase (SOD, EC 1.15.1.1) activity was measured by the inhibition of auto-oxidation of pyrogallol at 420 nm15. Glutathione reductase16 (GR, EC 1.6.4.2) and glutathione peroxidase17 (GPx, EC 1.11.1.9) activities were monitored using NADPH at 340 nm. Glutathione-s-transferase (GST; EC 2.5.1.18) activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB)18.

Statistical analysis
Results were expressed as mean ± SEM (standard error). Statistical significance was determined by Student’s ‘t’ test for unpaired data. The values of significance were evaluated with ‘P’ values. The differences were considered significant at P<0.05.

Results
In the present study, group I served as normal control, while group II served as experimental control. Preventive and curative effects of silybin and ascorbic acid on percentage change in body wt of animals, in relation to initial wt are shown in Fig. 1. When rats were simultaneously exposed to ethanol with silybin or ascorbic acid, 30% and 35% increase in body wt were found, respectively. After ethanol treatment for 4 wks, in the follow-up treatment with silybin or
ascorbic acid for another 4 weeks, 28% and 33% increase in body wt was found, while abstination showed 26% increase in body wt. No significant change was observed in the relative wt (g/100 g body wt) of liver of different treatments (Fig. 2).

The levels of ascorbic acid, TBARS, GSH content, and catalase, SOD, GPx, GR, and GST activities in the liver homogenate are presented in Table 1. No significant change was observed in ascorbic acid content. The level of lipid peroxidation (LPO) in the liver of all ethanol-treated groups was significantly higher than the control group. TBARS level was reduced by 10.3% and 16.9% during curative treatment with silybin and ascorbic acid, respectively.

**Table 1**—Effect of silybin and ascorbic acid on ascorbic acid content, TBARS and GSH levels and catalase, SOD, GPx and GST activities in liver homogenate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ascorbic acid&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TBARS&lt;sup&gt;b&lt;/sup&gt;</th>
<th>GSH&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Catalase&lt;sup&gt;d&lt;/sup&gt;</th>
<th>SOD&lt;sup&gt;e&lt;/sup&gt;</th>
<th>GR&lt;sup&gt;f&lt;/sup&gt;</th>
<th>GPx&lt;sup&gt;g&lt;/sup&gt;</th>
<th>GST&lt;sup&gt;h&lt;/sup&gt;</th>
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<tr>
<td>Control</td>
<td>3.02± 0.18</td>
<td>0.638± 0.015</td>
<td>4.12± 0.23</td>
<td>41.93± 0.66</td>
<td>5.88± 0.15</td>
<td>1.34± 0.15</td>
<td>2.48± 0.11</td>
<td>6.75± 0.11</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2.42± 0.43</td>
<td>0.893± 0.023</td>
<td>2.43± 0.18</td>
<td>29.58± 0.83</td>
<td>9.13± 0.21</td>
<td>0.84± 0.15</td>
<td>1.58± 0.31</td>
<td>12.78± 0.31</td>
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<tr>
<td>Abstination</td>
<td>2.49± 0.26</td>
<td>0.82± 0.048</td>
<td>2.72± 0.24</td>
<td>31.78± 1.78</td>
<td>8.42± 0.73</td>
<td>3.23± 0.16</td>
<td>6.71± 0.06</td>
<td>10.06± 0.06</td>
</tr>
<tr>
<td>Silybin Curative</td>
<td>2.52± 0.17</td>
<td>0.801± 0.14</td>
<td>2.88± 0.14</td>
<td>32.45± 1.43</td>
<td>8.22± 0.66</td>
<td>36.74± 1.76</td>
<td>63.69± 0.63</td>
<td>8.98± 0.63</td>
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<tr>
<td>Silybin Preventive</td>
<td>2.57± 0.19</td>
<td>0.793± 0.18</td>
<td>3.08± 0.18</td>
<td>33.69± 1.12</td>
<td>8.08± 0.58</td>
<td>37.26± 2.43</td>
<td>65.24± 3.26</td>
<td>8.87± 0.83</td>
</tr>
<tr>
<td>Ascorbic acid Curative</td>
<td>2.63± 0.14</td>
<td>0.742± 0.053</td>
<td>3.31± 0.26</td>
<td>34.32± 1.54</td>
<td>7.64± 0.58</td>
<td>42.74± 2.62</td>
<td>66.76± 3.98</td>
<td>8.23± 0.56</td>
</tr>
<tr>
<td>Ascorbic acid Preventive</td>
<td>2.71± 0.24</td>
<td>0.721± 0.046</td>
<td>3.48± 0.33</td>
<td>35.46± 1.98</td>
<td>7.42± 0.83</td>
<td>43.25± 2.83</td>
<td>68.87± 4.13</td>
<td>8.12± 0.46</td>
</tr>
</tbody>
</table>

<sup>a</sup>P < 0.05 compared to normal healthy control. <sup>b</sup>P < 0.05 compared to ethanol-treated group. <sup>c</sup>P < 0.05 compared to abstention group.

**Fig. 1**—Percent increase in body weight of control and other groups of rats exposed to different treatments [Values are mean ± SEM of 6 rats. *P < 0.05 compared to normal healthy control. # P < 0.05 compared to ethanol-treated group. @ P < 0.05 compared to abstention group]

**Fig. 2**—Relative weights of liver of control and other groups of rats exposed to different treatments [Values are expressed as mean of 6 rats. No significant change was observed in experimental groups, compared to control or ethanol-treated groups]
and under preventive treatment by 11.2% and 19.3% in comparison to ethanol-fed group. While during abstinence, reduction in TBARS level was 8.2%. The GSH content of liver homogenate of different groups was found to be significantly lower than the control group. GSH level was elevated by 26.7% and 43.2%, respectively during preventive treatment with silybin and ascorbic acid, when compared with ethanol-fed rats. Although similar trend was observed in curative treatment, preventive treatment showed better result.

The catalase activity of liver homogenate of silybin and ascorbic acid-treated groups was lower than the control group and higher, when compared with the ethanol-treated group (Table 1). On the other hand, reverse trend was observed in SOD activity. Glutathione reductase (GR) and glutathione peroxidase (GPx) activities of the liver of all the tested groups were found to be significantly lower than that of control group, and higher in comparison to ethanol-treated group (Table 1). In comparison to ethanol-fed group, GR level increased by 29.3% and 50.4%, respectively during curative treatment; and 31.1% and 52.8%, respectively during preventive treatment with silybin and ascorbic acid, whereas during abstention increase was 16.9%.

GPx level was raised by 10.53% and 15.86%, respectively during curative treatment, and 13.2% and 19.5%, respectively during preventive treatment with silybin and ascorbic acid, when compared with ethanol-fed rats.

Liver GST activity of all the tested groups was found to be significantly higher than that of control group and lower than ethanol-treated group. GST level was reduced by 29.7% and 35.6% respectively during curative treatment with silybin and ascorbic acid, whereas under preventive treatment reduced by 30.6% and 36.4%, in comparison to ethanol-fed groups. During abstention reduction in GST level was 22.9%. However, silybin and ascorbic acid-treated groups showed significantly lower values, in comparison to the abstention group.

Discussion

In the present study, an attempt has been made to validate hepatoprotective activity of silybin and ascorbic acid against ethanol-induced liver injury in rats. The study was carried out both on preventive and curative groups. A dose of 1.6 g ethanol/kg body wt/day for 4 wks was used to induce maximum liver damage, which could be reversed, as observed by our previous study. Rats that consumed 1.6 g ethanol/kg body wt/day for 4 wks showed a lower increase in body wt and lower liver wt, due essentially to the reduced adipose tissue. The animals with ALD have shown the impaired liver regeneration. In the present study, the regeneration was observed after administration of ethanol (1.6 g/kg body wt/day for 4 wks), followed by abstinence from ethanol for next 4 wks. Silybin and ascorbic acid exhibited an ability to counteract the ethanol-induced changes in the body wt and biochemical parameters in preventive and curative treatments in varying degree.

Ethanol consumption is associated with increased hepatic lipid peroxide content. Free radical formation results in increased lipid peroxidation, which exacerbates membrane injury. One of the proposed mechanisms of ethanol-induced toxicity is the membrane damage due to the direct effect of LPO. In the present study, TBARS were found increased in the ethanol-fed rats. Silybin and ascorbic acid reversed this level significantly, suggesting that they offered some protection against LPO. Oxidative stress is reflected by many factors. Ethanol-induced toxicity is protected by ascorbic acid. In the present study, ethanol administration decreased ascorbate level in the liver.

GSH has been postulated to be an important pathogenic factor in alcoholic liver injury. The depletion in hepatic GSH sensitizes the liver to oxidative injury and sets up a vicious cycle. Hepatic GSH has an especially important relationship with lipid peroxidation, because of its ability to bind with free radicals that may initiate peroxidation. The tissue GSH concentration reflects its potential for detoxification and is critical in preserving the proper cellular redox balance for its role as a cellular protectant. Increased levels of GSH elicit a protective response against the toxic manifestations of chemicals, particularly those involving oxidative stress. Chronic ingestion of ethanol resulted in a significant depletion of GSH level in liver. Both silybin and ascorbic acid treatments showed hepatoprotective properties, by preventing GSH depletion, due to ethanol treatment. Though abstinence from alcohol helped in increasing GSH level, but ascorbic acid-treated rats showed best result. It was also observed earlier that vitamin C supplementation decreased endogenous oxidative damage in guinea pigs under stressful condition.
Interestingly, we observed that silybin could regulate oxidative stress produced in the liver by ethanol. Silymarin or silibinin was able to protect animals from rats with acute intoxication caused by ethanol, and the antioxidant effect of silybin was observed in reducing the LPO. Enhancing the antioxidant capacity and also by prevention of ethanol-induced oxidative stress by study also suggested that ascorbic acid helps in the activities of scavenging enzymes. The present study reported that supplementation of a 25 mg ascorbic acid/kg body wt/day for 4 wks exposure, might be due to loss of NADPH, generation of superoxide, increased activity of LPO or combination of all. Different treatments increased its activity. Evidences suggest that intermediates of oxygen reduction may, in fact, be associated with the development of ALD.

Glutathione reductase (GR) is responsible with the maintenance of cellular level of GSH, by affecting fast reduction of oxidized glutathione to reduced form. Chronic ingestion of ethanol resulted in a significant decrease in GPx activity in liver, possibly due to free radical-dependent inactivation of the enzyme or depletion of co-substrates i.e., GSH and NADPH. GST plays an essential role in liver by eliminating toxic compounds by conjugating them with GSH. Increased GST and decreased GR activities, followed by thiol depletion are important factors sustaining a pathogenic role for oxidative stress. In the present study, supplementation of silybin or ascorbic acid as curative or preventive treatment could partly reverse these enzyme levels, due to oxidative stress.

Ascorbic acid protects lipids against peroxidation. It scavenges oxygen-free radical and is converted to dehydroascorbate. In animal tissue, the dehydroascorbate is reduced to ascorbate by GSH, resulting in recycling of ascorbate. Earlier, it was reported that supplementation of a 25 mg ascorbic acid/100 g body wt along with ethanol reduced the LPO products in the liver of guinea pigs and enhanced the activities of scavenging enzymes. The present study also suggested that ascorbic acid helps in the prevention of ethanol-induced oxidative stress by enhancing the antioxidant capacity and also by reducing the LPO.

The antioxidant effect of silybin was observed in rats with acute intoxication caused by ethanol, and silymarin or silibinin was able to protect animals from oxidative stress produced in the liver by ethanol. Interestingly, we observed that silybin could regulate GSH content in the liver. Earlier study also demonstrated a significant increase in the amount of the GSH contained in the liver, intestine and stomach after treatment with silibinin intravenously or silymarin intraperitoneally, whereas no change was observed in the lungs, spleen and kidneys of rats. Silymarin appears to act as an antioxidant, because it acts as a scavenger of the free radicals that induce LPO, and it also influences enzyme systems associated with GSH and SOD.

The hepatoprotective effect of silymarin is possibly due to its activity against LPO (as a result of free radical scavenging), the ability to increase the cellular content of GSH, the ability to regulate membrane permeability and to increase membrane stability in the presence of xenobiotic damage, capacity to regulate nuclear expression by means of a steroid-like effect, and inhibition of the transformation of stellate hepatocytes into myofibroblasts, which are responsible for the deposition of collagen fibers leading to cirrhosis. Furthermore, silymarin and silibinin, by interacting with the lipid component of cell membranes, can influence their chemical and physical properties.

But, the potential benefit of silybin in the treatment of liver diseases remained a controversial issue. A previous study showed that silymarin successfully opposed alcoholic cirrhosis in baboons. It was also observed to exert hepatoprotective activity and improved liver function in alcoholic patients. However, in another study, silymarin showed no considerable efficacy in alcoholic cirrhosis.

In conclusion, the present study showed that silybin (200 mg/kg body wt/day) supplementation had beneficial effect, in the therapy of ethanol-induced liver damage, compared to abstinence from alcohol, but was not as effective as supplementation of mega dose (1 g/kg body wt/day) of ascorbic acid. Preventive measures were more effective than curative treatment.

References

9 Package of Practices Recommendation (2001) Veterinary & Animal Husbandry Department, Kerala Agriculture University, Mannuthy, Thrissur
11 Roe J H & Kuether C A (1943) J Biol Chem 147, 399-401
12 Sinnhuber R O, Yu T C & Yu T C (1958) Food Res 23, 626-630
13 Ellman G L (1959) Arch Biochem Biophys 32, 70-77
17 Paglia D E & Valentine W N (1967) J Lab Clin Med 70, 158-159
27 Husain K & Somani S M (1997) J Appl Toxicol 17, 189-194