

# Silymarin modulates the oxidant–antioxidant imbalance during diethylnitrosamine induced oxidative stress in rats

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## Abstract

Oxidative stress is a common mechanism contributing to initiation and progression of hepatic damage in a variety of liver disorders. Hence, there is a great demand for the development of agents with potent antioxidant effect. The aim of the present investigation is to evaluate the efficacy of silymarin as a hepatoprotective and an antioxidant against diethylnitrosamine induced hepatocellular damage. Single intraperitoneal administration of diethylnitrosamine (200 mg/kg) to rats resulted in significantly elevated levels of serum aspartate transaminase (AST) and alanine transaminase (ALT), which is indicative of hepatocellular damage. Diethylnitrosamine induced oxidative stress was confirmed by elevated levels of lipid peroxidation and decreased levels of superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase (GR) and glutathione-S-transferase (GST) in the liver tissue. The status of non-enzymic antioxidants like, vitamin-C, vitamin-E and reduced glutathione (GSH) were also found to be decreased in diethylnitrosamine administered rats. Further, the status of membrane bound ATPases was also altered indicating hepatocellular membrane damage. Posttreatment with the silymarin (50 mg/kg) orally for 30 days significantly reversed the diethylnitrosamine induced alterations in the liver tissue and offered almost complete protection. The results from the present study indicate that silymarin exhibits good hepatoprotective and antioxidant potential against diethylnitrosamine induced hepatocellular damage in rats.

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**Keywords:** Silymarin; Diethylnitrosamine; Antioxidant; Hepatotoxicity; Lipid peroxidation

## 1. Introduction

Diethylnitrosamine is an *N*-nitroso alkyl compound, categorized as a potent hepatotoxin and hepatocarcinogen in experimental animals, producing reproducible tumors after repeated administration (Jose et al., 1998). The main cause for concern is that diethylnitrosamine is found in a wide variety of foods like cheese, soybean, smoked, salted and dried fish, cured meat and alcoholic beverages (Liao et al., 2001). Metabolism of certain therapeutic drugs is also reported to produce diethylnitrosamine (Akintonwa, 1985). It is also found in tobacco smoke at a concentration ranging from 1 to 28 ng/cigarette and in baby bottle nipples at a level of 10 ppb (IARC, 1972). Diethylnitrosamine is reported to undergo metabolic activation by

cytochrome P450 enzymes to form reactive electrophiles which cause oxidative stress leading to cytotoxicity, mutagenicity and carcinogenicity (Archer, 1989). The detection of diethylnitrosamine in commonly consumed food products makes the human population vulnerable to its exposure. This constraint underscores the need for the development of novel hepatoprotective drug with potent antioxidant activity. Various plants and plant derived products have been tested and found to be effective against diethylnitrosamine induced hepatocarcinogenesis and hepatotoxicity (Ahmed et al., 2001). As oxidative stress plays a central role in diethylnitrosamine induced hepatotoxicity, the use of antioxidants would offer better protection to counteract liver damage (Vitaglione et al., 2004). In light of these observations, it was decided to evaluate the efficacy of silymarin, a plant flavanoid, as an antioxidant against diethylnitrosamine induced hepatocellular damage.

Silymarin, a known standardized extract obtained from seeds of *Silybum marianum* (Family: Composite) is widely used in

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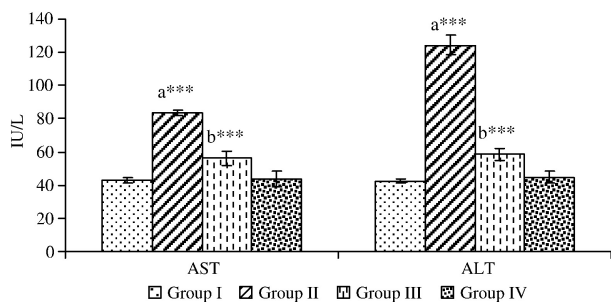


Fig. 1. Effect of silymarin on AST and ALT levels in serum during diethylnitrosamine induced hepatotoxicity. Results are given as mean±S.E.M. for six rats. Comparisons are made between: a — control rats (Group I); b — DEN treated rats (Group II). \*\*\*  $P < 0.001$ .

treatment of liver diseases of varying origin (El-Samaligy et al., 2006). Silymarin is a purified extract from milk thistle *Silybum marianum* composed of a mixture of four isomeric flavonolignans: silibinin (its main, active component), isosilibinin, silydianin and silychristin (Crocenzi and Roma, 2006). The plant has been used since 4th century BC for the treatment of plague and congestive conditions of the liver and spleen (Choksi et al., 2000). Seeds of *S. marianum* have been used for more than 2000 years to treat liver and gall bladder disorders, including hepatitis, cirrhosis and jaundice and to protect the liver against poisoning from chemicals, environmental toxins, snake bites, insect stings, mushroom poisoning and alcohol (Kren and Walterova, 2005). Silymarin is widely used for protection against various hepatobiliary problems in Europe (Flora et al., 1998). It is also reported to offer protection against chemical hepatotoxins such as  $\text{CCl}_4$  (Muriel and Mourelle, 1990), acetaminophen (Muriel et al., 1992), phalloidin, galactosamine and thioacetamide (Fraschini et al., 2002) and alcoholic liver diseases (Feher et al., 1989). Due to its proven hepatoprotective and antioxidant properties, silymarin is being used as a standard agent for comparison in the evaluation of hepatoprotective effects of plant principles (Dhiman and Chawla, 2005).

The present investigation was carried out with the objective of evaluating the efficacy of the plant flavanoid silymarin in

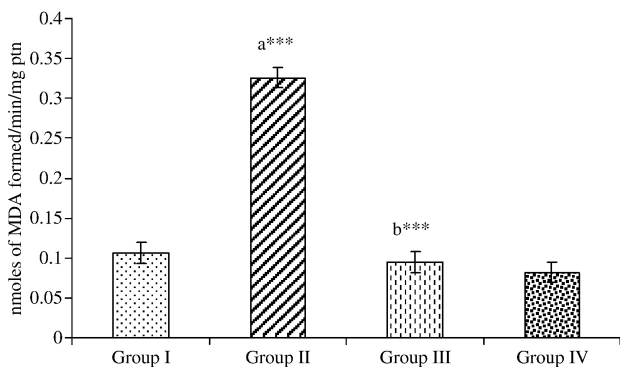


Fig. 2. Effect of silymarin on lipid peroxidation in liver tissue of experimental animals during diethylnitrosamine induced hepatotoxicity. Results are given as mean±S.E.M. for six rats. Comparisons are made between: a — control rats (Group I); b — DEN treated rats (Group II). \*\*\*  $P < 0.001$ .

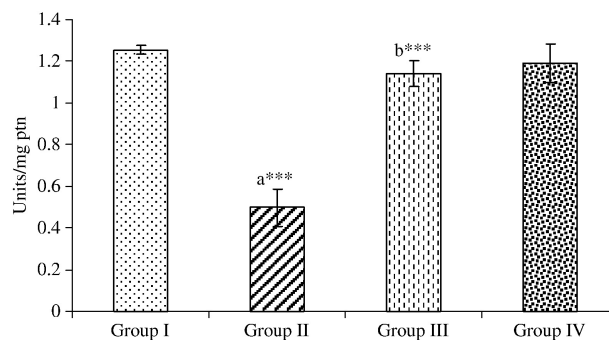


Fig. 3. Effect of silymarin posttreatment on the activity of superoxide dismutase in liver tissue of experimental animals during diethylnitrosamine induced hepatotoxicity. Results are given as mean±S.E.M. for six rats. Comparisons are made between: a — control rats (Group I); b — DEN treated rats (Group II). \*\*\*  $P < 0.001$ .

maintaining the balance in the oxidant–antioxidant status during diethylnitrosamine induced hepatic oxidative stress in Wistar albino rats.

## 2. Materials and methods

### 2.1. Animals

Healthy male Wistar albino rats weighing  $200 \pm 10$  g purchased from Tamil Nadu Veterinary and Animal Sciences University, Chennai, were used for this study. Animals were housed in poly propylene cages and were provided certified rodent pellet diet and water *ad libitum*. They were maintained at  $25^\circ\text{C}$  with 12 h light and dark cycle. All animal experiments were performed in accordance with the strict guidelines prescribed by the Institutional Animal Ethical Committee (IAEC) after getting necessary approval.

### 2.2. Chemicals

Diethylnitrosamine and 1,1,3,3, tetraethoxypropane were purchased from Sigma Chemical Company, USA. 5-5'-dithio-bis-2-nitrobenzoic acid (DTNB) and 1-chloro 2, 4-dinitrobenzene (CDNB) were purchased from SISCO Research

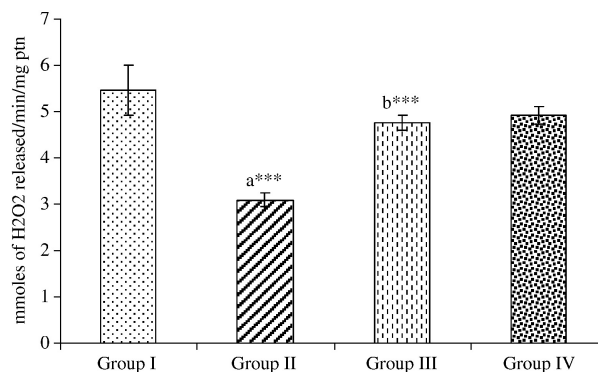


Fig. 4. Effect of silymarin posttreatment on the activity of catalase in liver tissue of experimental animals during diethylnitrosamine induced hepatotoxicity. Results are given as mean±S.E.M. for six rats. Comparisons are made between: a — control rats (Group I); b — DEN treated rats (Group II). \*\*\*  $P < 0.001$ .

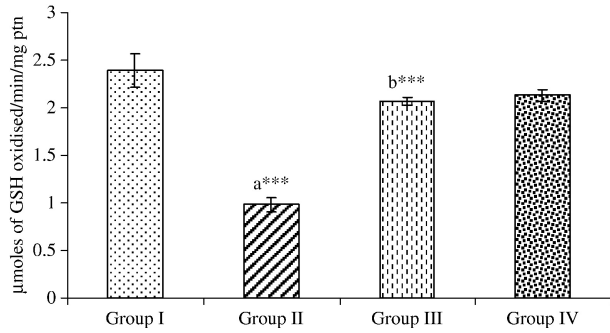


Fig. 5. Effect of silymarin posttreatment on the levels of glutathione peroxidase in liver tissue of experimental animals during diethylnitrosamine induced hepatotoxicity. Results are given as mean  $\pm$  S.E.M. for six rats. Comparisons are made between: a — control rats (Group I); b — DEN treated rats (Group II). \*\*\*  $P < 0.001$ .

Laboratories, Chennai, India. Silymarin was obtained as *gratis* from Central Drug Research Institute (CDRI), Lucknow, India. All the other chemicals used were of analytical grade and were purchased locally.

### 2.3. Experimental design

Rats were divided into 4 groups with 6 animals in each group. The experimental design was as follows: Group I rats served as controls and were treated with saline (i.p.) on day 0 and propylene glycol (orally) for 30 days. Group II rats were administered a single dose of diethylnitrosamine (200 mg/kg b. w., i.p.) in saline (Goldsworthy and Hanigan, 1986) on day 0 and propylene glycol (orally) from day 1 to 30. Group III rats were administered diethylnitrosamine (200 mg/kg b.w., i.p.) in saline on day 0 followed by silymarin (50 mg/kg b.w., p.o.) in propylene glycol (Fraschini et al., 2002) from day 1 till day 30. Group IV rats were treated with saline (i.p.) on day 0 and silymarin (50 mg/kg b.w., p.o.) in propylene glycol from day 1 till day 30.

At the end of experimental period, animals were subjected to mild ether anaesthesia, blood was collected from retro orbital plexus and the serum was separated by centrifugation at 3000 rpm for 15 min at 4 °C. Animals were sacrificed by cervical decapitation and the liver was excised, washed in ice

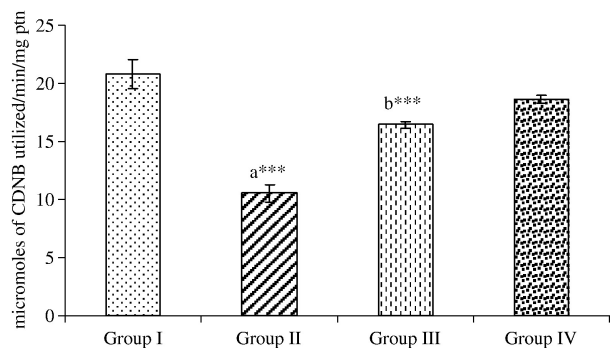


Fig. 6. Effect of silymarin posttreatment on the levels of glutathione-S-transferase in liver tissue of experimental animals during diethylnitrosamine induced hepatotoxicity. Results are given as mean  $\pm$  S.E.M. for six rats. Comparisons are made between: a — control rats (Group I); b — DEN treated rats (Group II). \*\*\*  $P < 0.001$ .

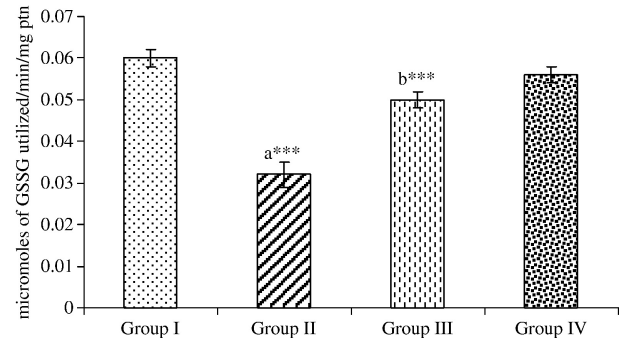


Fig. 7. Effect of silymarin posttreatment on the levels of glutathione reductase in liver tissue of experimental animals during diethylnitrosamine induced hepatotoxicity. Results are given as mean  $\pm$  S.E.M. for six rats. Comparisons are made between: a — control rats (Group I); b — DEN treated rats (Group II). \*\*\*  $P < 0.001$ .

cold saline and blotted to dryness. A 1% homogenate of the liver tissue was prepared in Tris-HCl buffer (0.1 M; pH 7.4), centrifuged at 1000 rpm for 10 min at 4 °C to remove the cell debris. The clear supernatant used for further biochemical assays. Aspartate and alanine transaminases (AST and ALT) were assayed according to the method of Wooten (1964), lipid peroxidation was determined in the liver tissue as described by Ohkawa et al. (1979), superoxide dismutase (SOD) according to Marklund and Marklund (1974) and catalase according to the method of Sinha (1972). The hepatic glutathione (GSH) content was estimated by the method of Ellman (1959) with little modification (Beutler et al., 1963). The activity of glutathione-S-transferase (GST) in the liver tissue was estimated by the method of Habig et al. (1974). The activity of glutathione peroxidase in the liver was estimated by the methods of Rotruck et al. (1973) and Beutler et al. (1963). The activity of hepatic glutathione reductase (GR) was estimated in the liver tissue by the method of Mize and Langdon (1962). The vitamin-C (ascorbic acid) content in the liver tissue was determined according to the method of Omaye et al. (1979). The vitamin-E ( $\alpha$ -tocopherol) content in the liver tissue was estimated as detailed in Varley et al. (1976). The activity of  $\text{Na}^+/\text{K}^+$  ATPase in the liver tissue was estimated by Bonting (1970),  $\text{Ca}^{2+}$  ATPase as described by Hjerten and Pan (1983) and  $\text{Mg}^{2+}$

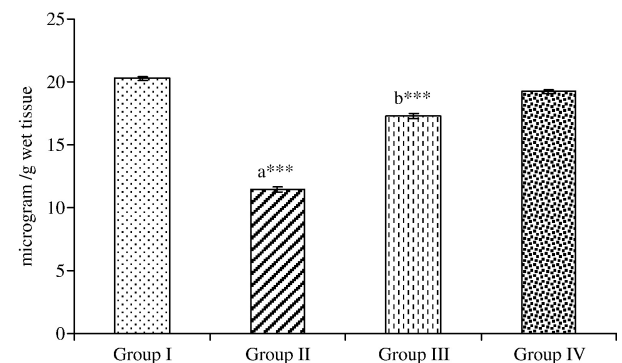


Fig. 8. Effect of silymarin posttreatment on the levels of GSH in liver tissue of experimental animals during diethylnitrosamine induced hepatotoxicity. Results are given as mean  $\pm$  S.E.M. for six rats. Comparisons are made between: a — control rats (Group I); b — DEN treated rats (Group II). \*\*\*  $P < 0.001$ .

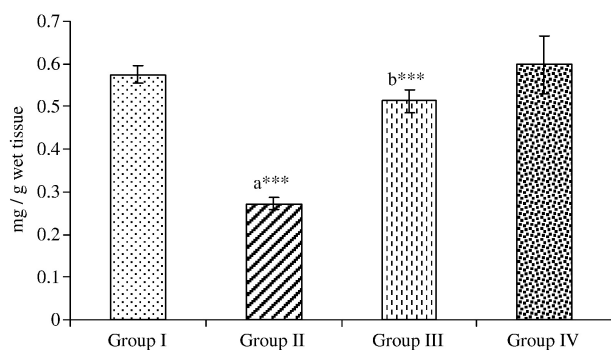


Fig. 9. Effect of silymarin posttreatment on the levels of vitamin-C in liver tissue of experimental animals during diethylnitrosamine induced hepatotoxicity. Results are given as mean  $\pm$  S.E.M. for six rats. Comparisons are made between: a — control rats (Group I); b — DEN treated rats (Group II). \*\*\*  $P < 0.001$ .

ATPase by the method of Ohnishi et al. (1982) in which the liberated phosphate was estimated according to the method of Fiske and Subbarow (1925). Protein was estimated as described by Lowry et al. (1951).

#### 2.4. Statistical analysis

The data obtained was subjected to One way ANOVA and Tukey's multiple comparison test was performed using SPSS statistical package (Version 7.5). Values are expressed as mean  $\pm$  S.E.M.  $P$  value  $< 0.05$  was considered significant.

### 3. Result

Fig. 1 shows the status of serum AST and ALT in control and experimental animals. Diethylnitrosamine (Group II) induced hepatotoxicity is shown by a 2 fold increase in the activity of AST and a 3 fold increase in ALT in the serum of rats as compared to saline treated normal controls (Group I). This increased activity of AST and ALT in serum due to diethylnitrosamine challenge was significantly decreased on posttreatment with silymarin (Group III) for 30 days.

The changes in the levels of lipid peroxidation in the liver tissue of control and experimental rats are illustrated in Fig. 2. Lipid peroxidation level was significantly elevated (about 3 fold) in diethylnitrosamine treated rats when compared with

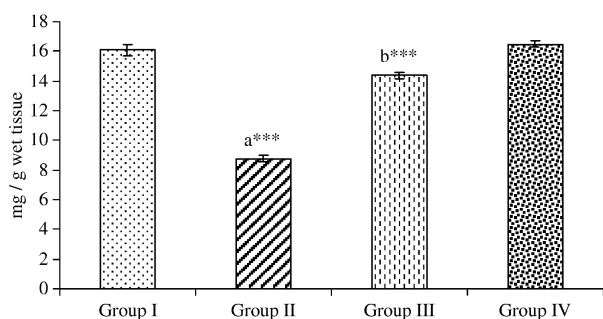


Fig. 10. Effect of silymarin posttreatment on the levels of vitamin-E in liver tissue of experimental animals during diethylnitrosamine induced hepatotoxicity. Results are given as mean  $\pm$  S.E.M. for six rats. Comparisons are made between: a — control rats (Group I); b — DEN treated rats (Group II). \*\*\*  $P < 0.001$ .

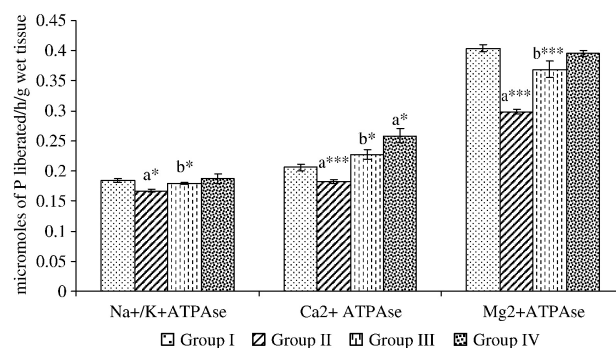


Fig. 11. Effect of silymarin posttreatment on the activities of ATPases in liver tissue of experimental animals during diethylnitrosamine induced hepatotoxicity. Results are given as mean  $\pm$  S.E.M. for six rats. Comparisons are made between: a — control rats (Group I); b — DEN treated rats (Group II). \*\*\*  $P < 0.001$  and \*  $P < 0.05$ .

control rats. Posttreatment with silymarin for 30 days offered significant protection against diethylnitrosamine induced elevation in lipid peroxidation as evidenced by a significant fall in its levels.

The changes in the activities of enzymic antioxidants namely SOD, catalase and glutathione peroxidase in liver of control and experimental animals are shown in Figs. 3, 4 and 5 respectively. A significant decrease in the activities of these radical scavengers was noted after single dose administration of diethylnitrosamine. Upon administration of silymarin for 30 days, the activities of enzymic antioxidants were significantly reversed to near normalcy.

The activities of GST and GR and the levels of GSH in the liver tissue were decreased significantly by about 50% of control value in diethylnitrosamine treated rats (Figs. 6–8). Posttreatment of silymarin for 30 days significantly reversed the fall in the levels of the above parameters. The levels of antioxidant vitamins, vitamin-C and vitamin-E were also decreased in rats treated with diethylnitrosamine as compared to control (Figs. 9 and 10). Silymarin treatment for 30 days caused a significant reversal of the fall in the levels of vitamin-C and vitamin-E in the liver tissue.

Activities of Na<sup>+</sup>K<sup>+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup> ATPases were significantly decreased in the liver tissue of diethylnitrosamine alone treated rats as compared to normal controls (Fig. 11). Posttreatment with silymarin (Group III) significantly prevented the above decrease in the activities of all ATPases induced by diethylnitrosamine alone treatment. Silymarin alone treatment (Group IV) caused a mild increase in the activity of Ca<sup>2+</sup> ATPase but did not cause any alteration in other parameters investigated in the serum and liver tissue compared to normal control.

### 4. Discussion

Diethylnitrosamine, one of the most important environmental carcinogen, has been suggested to cause the generation of reactive oxygen species (ROS) resulting in oxidative stress and cellular injury (Bartsch et al., 1989). As liver is the main site of diethylnitrosamine metabolism, the production of ROS in liver may be responsible for its carcinogenic effects (Bansal et al.,

2005). The involvement of oxidative stress in diethylnitrosamine induced hepatotoxicity and carcinogenicity underscores the need for development of novel compound with potent antioxidant activity. In this study, diethylnitrosamine administration to rats lead to a marked elevation in the levels of serum AST and ALT which is indicative of hepatocellular damage. This might be due to the possible release of these enzymes from the cytoplasm, into the blood circulation rapidly after rupture of the plasma membrane and cellular damage. Serum AST and ALT are the most sensitive markers employed in the diagnosis of hepatic damage because they are cytoplasmic in location and hence released into the circulation after cellular damage (Wroblewski, 1959; Sallie et al., 1991). Several studies have reported similar elevation in the activities of serum AST and ALT during diethylnitrosamine administration (Bansal et al., 2000). Treatment with silymarin significantly reduced the activities of the above marker enzymes in diethylnitrosamine treated rats. This indicates that silymarin tends to prevent liver damage by maintaining the integrity of the plasma membrane, thereby suppressing the leakage of enzymes through membranes, exhibiting hepatoprotective activity. This might be the reason for the restoration in the activities of the marker enzymes during administration of silymarin.

Oxidative damage in a cell or tissue occurs when the concentration of reactive oxygen species ( $O_2\cdot^-$ ,  $H_2O_2$ , and  $OH\cdot$ ) generated exceeds the antioxidant capability of the cell (Sies, 1991). The status of lipid peroxidation as well as altered levels of certain endogenous radical scavengers is taken as direct evidence for oxidative stress (Khan, 2006). Free radical scavenging enzymes like SOD and catalase protect the biological systems from oxidative stress. The SOD dismutates superoxide radicals ( $O_2\cdot^-$ ) into hydrogen peroxide ( $H_2O_2$ ) and  $O_2$  (Fridovich, 1986). Catalase further detoxifies  $H_2O_2$  into  $H_2O$  and  $O_2$  (Murray et al., 2003). Glutathione peroxidase also functions in detoxifying  $H_2O_2$  similar to catalase. Thus, SOD, catalase and glutathione peroxidase act mutually and constitute the enzymic antioxidative defense mechanism against reactive oxygen species (Bhattacharjee and Sil, 2006). The decrease in the activities of these enzymes in the present study could be attributed to the excessive utilization of these enzymes in inactivating the free radicals generated during the metabolism of diethylnitrosamine. This is further substantiated by an elevation in the levels of lipid peroxidation. Similar reports have shown an elevation in the status of lipid peroxidation in the liver during diethylnitrosamine treatment (Nakae et al., 1997; Sanchez-Perez et al., 2005) and our results are in accordance with these reports. Restoration in the levels of lipid peroxidation after administration of silymarin could be related to its ability to scavenge reactive oxygen species, thus preventing further damage to membrane lipids. Our results are in line with previous studies by Ramakrishnan et al. (2006) who have shown that silymarin exhibits excellent antioxidant property. Therefore this property of silymarin might have resulted in the recouplement in the activities of the above antioxidant enzymes to normalcy.

Excessive liver damage and oxidative stress caused by diethylnitrosamine depleted the levels of non-enzymic antioxidants like GSH, vitamin-C and vitamin-E in our study. Non-enzymic antioxidants like vitamin-C and E act synergistically to scavenge the free radicals formed in the biological system. GSH

acts synergistically with vitamin-E in inhibiting oxidative stress and acts against lipid peroxidation (Chaudiere, 1994). Vitamin-C also scavenges and detoxifies free radicals in combination with vitamin-E and glutathione (George, 2003). It plays a vital role by regenerating the reduced form of vitamin-E and preventing the formation of excessive free radicals (Das, 1994). The decreased levels of these antioxidant vitamins and GSH observed during diethylnitrosamine administration might be due to the excessive utilization of these vitamins in scavenging the free radicals formed during the metabolism of diethylnitrosamine. Silymarin treatment effectively restored the depleted levels of these non-enzymic antioxidants caused by diethylnitrosamine. Silymarin has been reported to maintain the GSH homeostasis in the system (Fraschini et al., 2002) and this might be the reason for elevated glutathione levels observed during silymarin treatment. Increase in GSH levels in turn contributes to the recycling of other antioxidants such as vitamin-E and vitamin-C (Exner et al., 2000). This shows that silymarin maintains the levels of antioxidant vitamins by maintaining GSH homeostasis, thereby protecting the cells from further oxidative stress.

A significant decrease in the activities of GSH dependent enzymes, GST and GR was observed in diethylnitrosamine treated rats, which may be due to the decreased expression of these antioxidants during hepatocellular damage. Further, the decreased levels of cellular GSH might have also caused a reduction in their activities as GSH is a vital co-factor for these enzymes. Our observations are in accordance with the reports of Kweon et al. (2003) who demonstrated that diethylnitrosamine induced hepatocellular injury was escorted by a substantial fall in hepatic GSH level and glutathione peroxidase and GST activity, which improved on administration of antioxidants. It is likely that posttreatment of silymarin similarly maintains the activity of GR and GST in the liver by inhibiting lipid peroxidation and maintaining GSH levels. This is indicative of the potent antioxidant activity possessed by silymarin.

The membrane bound enzymes *i.e.*,  $Na^+/K^+$  ATPase,  $Ca^{2+}$  ATPase and  $Mg^{2+}$  ATPase are responsible for the transport of ions across cell membrane at the expense of ATP. Studies have shown that hepatic injury resulting from peroxidation of membrane lipids results in the alteration of structural and functional characteristics of the membrane, which affects the activities of these membrane bound ATPases. These enzymes are extremely sensitive to hydroperoxides and superoxide radicals (Jain and Shohet, 1981), which might be the reason for their decreased activities during diethylnitrosamine administration. This fact is further supported by the studies of Koizumi et al. (1995), who have reported disruption in calcium and potassium metabolism in liver of diethylnitrosamine treated rats. The increase in the activity of the above ATPases on posttreatment with silymarin could be due to the membrane stabilizing activity by preventing peroxidation of membrane lipids. Studies have shown that antioxidants like turmeric inhibit diethylnitrosamine induced hepatotoxicity (Thapliyal et al., 2003). Therefore, silymarin by virtue of its antioxidant role could have prevented damage to the hepatocytes and thereby maintained the membrane in a healthy state.

The results of the present investigation shows silymarin to be effective in scavenging the free radicals released during the

metabolism of diethylnitrosamine, which is evidenced by the decrease in the levels of lipid peroxidation after 30 days of administration of silymarin. The ability of silymarin in maintaining the membrane integrity is evidenced by the restoration of the activities of membrane bound ATPases, restoration in the activities of hepatic marker enzymes and antioxidant enzymes. Further, silymarin also maintained the status of glutathione dependent enzymes and antioxidant vitamins by virtue of its ability to regulate the cellular glutathione content (Valenzuela et al., 1989). In conclusion, our present investigation shows that the silymarin exhibits excellent hepatoprotective property by restoring the hepatic marker enzymes and antioxidant property by reversing the oxidant–antioxidant imbalance during diethylnitrosamine induced oxidative stress in rats.

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