

RESEARCH ARTICLE

# Silibinin potentially protects arsenic-induced oxidative hepatic dysfunction in rats

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## Abstract

Arsenic (As) compounds are reported as environmental toxicants and human carcinogens. Exposure to arsenic imposes a big health issue worldwide. Silibinin (SB) is a major flavonolignan compound of silimarin and is found in milk thistle of *Silybum marianum*. It has been reported that silibinin has antioxidant efficacy as metal chelators due to the orientation of its functional groups. However, it has not yet been explored in experimental animals. In view of this fact, the purpose of this study was to delineate the ameliorative role of silibinin against arsenic-induced hepatotoxicity in rats. Rats were orally treated with arsenic alone (5 mg/kg body weight (bw)/day) plus silibinin (75 mg/kg bw/day) for 4 weeks. Hepatotoxicity was evaluated by the increased activities of serum hepatospecific enzymes namely aspartate transaminase, alanine transaminase, alkaline phosphatase, gamma glutamyl transferase, lactate dehydrogenase and total bilirubin along with increased elevation of lipid peroxidative markers, thiobarbituric acid reactive substances, lipid hydroperoxides, protein carbonyl content and conjugated dienes. The toxic effect of arsenic was also indicated by significantly decreased activities of membrane bound ATPases, enzymatic antioxidants like superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, glutathione reductase and glucose-6-phosphate dehydrogenase along with nonenzymatic antioxidants like reduced glutathione, total sulfhydryl groups, vitamins C and E. Administration of silibinin exhibited a significant reversal of arsenic-induced toxicity in hepatic tissue. All these changes were supported by reduction of DNA damage in hepatocytes and histopathological observations of the liver. These results suggest that silibinin has a potential protective effect over arsenic-induced hepatotoxicity in rat.

**Keywords:** Arsenic, silibinin, hepatotoxicity, oxidative stress, comet assay, rats

## Introduction

Arsenic is ubiquitous in nature and its abundance ranks 20th in the Earth's crust, 14th in seawater and 12th in the human body. The largest source of arsenic is usually food, of which the main dietary forms are seafood, rice, mushrooms and poultry (Jones, 2007; Nepusz et al., 2009). Besides the natural sources, repeated uses of arsenic as herbicides, insecticides and rodenticides are drastically contaminating drinking water (Gupta et al., 2005). Groundwater contamination by arsenic and other metals has impacted severely on the health of the populations of various regions in the world. Some of the most profound examples of contamination by arsenic occur in Bangladesh and West Bengal, in India, where it has been discovered that almost 43 million people have been drinking water that is laden with arsenic (Chowdhury et al., 2000). Arsenic

is the first metalloid to be identified as a human carcinogen. In addition, several epidemiological studies have revealed that chronic exposure to arsenic has been linked with myriad of human diseases, such as diabetes, atherosclerosis, cardiovascular diseases and hyperkeratosis (Tchounwou et al., 2003). Since arsenic targets ubiquitous enzyme reactions, it affects nearly all organ systems in animals and humans (Guhmajumdar, 2005).

Literature studies have revealed that the liver is an important target organ for arsenic toxicity and its importance as an organ for arsenic biotransformation is well established in its enzymatic reactions (Santra et al., 1999). The exact cellular mechanisms by which arsenic produces hepatotoxicity *in vivo* are still unclear, but the advancement of research over the past decade demonstrated that oxidative stress is the key contributor

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in arsenic-induced hepatic injury as it is known to produce reactive oxygen species, namely superoxide ( $O_2^-$ ), hydroxyl ( $\cdot OH$ ), peroxy radicals ( $ROO\cdot$ ) and hydrogen peroxide ( $H_2O_2$ ; Liu et al., 2001). Furthermore, arsenic exposure was shown to depress the antioxidant defense system (Sharma et al., 2007) leading to the oxidative damage of cellular macromolecules including DNA, proteins and lipids (Shi et al., 2004) that cause damage at the membrane, cell and tissue levels which ultimately wreak havoc to the biological system (Wiseman and Halliwell, 1996). Oxidative stress in the liver may lead to hepatocellular injuries, hydropic, fatty degeneration, progressive fibrosis and more critical consequences. Many investigators have confirmed that arsenic induces hepatic oxidative stress and the use of antioxidants have recently been considered as therapeutic agents to counteract liver damages to protect the cellular machinery from peroxidative injury inflicted by reactive oxygen species and reactive nitrogen species (Vitaglione et al., 2004).

Silibinin is a major flavonolignan of silymarin (Figure 1), isolated from the seeds of milk thistle (*Silybum marianum* (L.) Gaertn.), and it has been traditionally used against various hepatic ailments (Saller et al., 2001) and cancer (Singh and Agarwal, 2002). Silibinin has already been reported to have a broad spectrum of pharmacological activities such as hepatoprotective (Ferenci, 1989), antioxidant (Saller et al., 2001), metal chelation (Pietrangolo et al., 1995), cardioprotective (Agoston et al., 2001), neuroprotective (Kittur et al., 2002) and a specific protective action on DNA damage (Yoo et al., 2004). Moreover, SB is the most biologically active component among the silimarin flavonolignans with regard to its antioxidant and hepatoprotective properties, it is concentrated in bile, achieving concentrations 60 times higher than that found in serum (Lorenz et al., 1984).

To our knowledge, no other biochemical investigations have so far been carried out and this is the first attempt to explore the hepatoprotective nature of silibinin in arsenic hepatotoxicity. Therefore, the purpose of this study was to investigate the possible protective role of silibinin against arsenic-induced oxidative hepatic damage and bring hepatic recovery in terms of biochemical, molecular and histological indices.

## Materials and methods

### Chemicals

Sodium arsenite ( $NaAsO_2$ ), silibinin, 1,1',3,3'-tetramethoxy propane, bovine serum albumin, Hank's

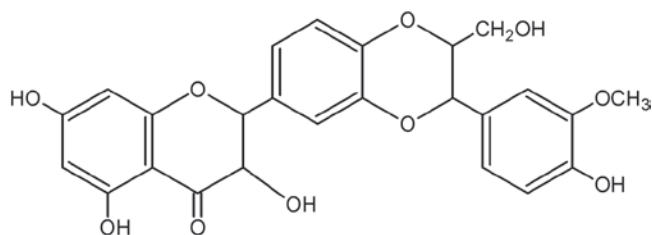


Figure 1. Chemical structure of silibinin ( $C_{25}H_{21}O_{10}$ ).

balanced salt solution, Ficol histopaque-1077, phosphate buffered saline and SYBR green-I were purchased from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals and solvents were of certified analytical grade and purchased from S.D. Fine Chemicals, Mumbai or Himedia Laboratories Pvt. Ltd., Mumbai, India. Reagent kits were obtained from span Diagnostics, Mumbai, India.

### Animals and diet

Healthy adult male albino rats of Wistar strain, bred and reared in Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College, Annamalai University were used for the experiment. Males were preferred to avoid complications of the oestrous cycle. Animals of equal weight (170–190 g) were selected and housed in polypropylene cages lined with husk and kept in a seminatural light/dark condition (12h light/12h dark). The animals had free access to water and were supplied with standard pellet diet (Amrut Laboratory Animal Feed, Pranav Agro Industries Ltd., Bangalore, India), constitution of protein (22.21%), fat (3.32%), fibre (3.11%), balanced with carbohydrates (>67%), vitamins and minerals. Animal handling and experimental procedures were approved by the Institutional Animal Ethics Committee, Annamalai University (Registration Number: 684/2010/CPCSEA) and the animals were cared in accordance with the "Guide for the care and use of laboratory animals" and "Committee for the purpose of control and supervision on experimental animals."

### Experimental design

In the present study,  $NaAsO_2$  was administered intragastrically at a dose of 5 mg/kg body weight/day for 4 weeks, which was 1/8 of the oral  $LD_{50}$  values in rats (North et al., 1997). Control group received the vehicles only, experimental rats were subdivided into two groups (2 and 3). Drug control group received the SB (dissolved in 0.5% of carboxy methyl cellulose, CMC) alone.

A pilot study was conducted with three different doses of SB (25, 50 and 75 mg/kg) to determine the dose-dependent effect of SB in As treated hepatotoxic rats. After 4 weeks of experiment, it was observed that SB treatment at the doses of 25, 50 and 75 mg/kg significantly ( $p < 0.05$ ) lowered the levels of serum transaminases, thiobarbituric acid reactive substances and elevated the levels of reduced glutathione in the liver of As intoxicated rats (data have not shown). 75 mg/kg of SB showed higher significant effect than the lower doses 25 and 50 mg/kg. Hence, we have chosen the highest dose (75 mg/kg) of SB for our study.

Group 1: ( $n=6$ ) Considered as negative controls where rats received daily normal saline and CMC solutions used as vehicles.

Group 2: ( $n=6$ ) Corresponding to As treated group where rats received daily  $NaAsO_2$  (5 mg/kg Bw) dissolved in saline solution.

Group 3: ( $n=6$ ) Rats received daily silibinin (SB) dissolved in CMC (75 mg/kg Bw) along with arsenic (5 mg/kg Bw).

Group 4: ( $n=6$ ) Considered as positive controls where rats received daily SB dissolved in CMC (75 mg/kg Bw).

All treatments were made by intragastric tube for 4 weeks. Food and water intake was recorded and rats were weighed every week. Forty-eight hours after the administration of the last dose, the animals were sacrificed by decapitation. Blood was collected in tubes for the separation of serum. Liver tissue was surgically removed, washed with cold physiological saline, cleared off adherent lipids and immediately transferred to ice-cold containers. Liver tissue (250 mg) was sliced into pieces and homogenized in appropriate buffer in cold condition (pH 7.0) to give 20% homogenate (w/v). The homogenate was centrifuged at 1000 rpm for 10 min at 0°C in cold centrifuge. The supernatant was separated and used for the assay of various biochemical estimations. For comet assay, liver was cut into small pieces and washed with Hank's balanced salt solution. The individual cell suspensions were obtained by enzymic digestion with collagenase at 37°C. After filtration on 100 and 40 mm mesh successively, the resulting cell suspensions were centrifuged for 10 min at 3000 rpm. The cell pellet was then suspended in phosphate buffered saline and used for the estimation of DNA damage.

## Biochemical assays

### Hepatic marker enzymes and bilirubin

The activities of serum aspartate aminotransferase (E.C. 2.6.1.1), alanine aminotransferase (E.C. 2.6.1.2), alkaline phosphatase (E.C.3.1.3.1), lactate dehydrogenase (E.C. 3.1.3.1) and total bilirubin were assayed using commercially available diagnostic kits (Sigma diagnostics (I) Pvt. Ltd., Baroda, India). Gamma glutamyl transferase (E.C. 2.3.2.2) activity was determined by the method of Rosalki et al. (1970) using  $\gamma$ -glutamyl-*p*-nitroanilide as substrate. Based on Van den Berg reaction, serum bilirubin was estimated by the method of Malloy and Evelyn (1937).

### Determination of blood $\delta$ -aminolevulinic acid dehydratase (ALAD)

The activity of blood  $\delta$ -aminolevulinic acid dehydratase (ALAD) was assayed according to the procedure of Berlin and Schaller (1974). Total volume of 0.2 mL of heparinized blood was mixed with 1.3 mL of distilled incubated for 10 min at 37°C for complete hemolysis. After adding 1 mL of standard ALA, the tubes were incubated for 60 min at 37°C. The reaction was stopped after 1 h by adding 1 mL of trichloroacetic acid (TCA). After centrifugation, equal volume of Ehrlich reagent (0.59 g of dimethylamino benzaldehyde in 12.5 mL glacial acetic acid + 6 mL perchloric acid + 1 mL 2.5% mercuric chloride) was added and the absorbance was recorded at 555 nm after 5 min. The values are expressed as nmol/min/mL.

### Determination of liver $\delta$ -aminolevulinic acid dehydratase (ALAD)

Liver  $\delta$ -aminolevulinic acid dehydratase (ALAD) was determined by following the method of Goering et al. (1988). 20% tissue homogenate (w/v) was prepared in 0.05 M Tris-HCL (pH 7.4) and 0.25 M sucrose. The incubation medium (0.3 mL) contained 4 mg protein, 50 mM glycine, 50 mM-sodium citrate, 25 mM-sodium monophosphate, 10 mM-magnesium chloride, 5 mM-pyridoxal 5-phosphate and 10 mM disodium EDTA. The mixture was incubated for 45 min at 37° C in darkness. The aminoketones formed were converted into pyrroles by condensation with acetyl acetone. The activity of the enzyme was determined by adding Ehrlich reagent and the absorbance was recorded at 555 nm after 5 min. The values are expressed as nmol/min/mg protein.

### Determination of lipid peroxidation and oxidative stress markers

Lipid peroxidation in liver was estimated spectrophotometrically by measuring thiobarbituric acid reactive substances and lipid hydroperoxides by the method of Niehius and Samuelson (1968), Jiang et al. (1992), respectively. Protein carbonyl content was determined by the method of Levine et al. (1999). The levels of conjugated dienes were assessed by the method of Rao and Racknagel (1968).

### Determination of nonenzymatic and enzymatic antioxidants

Reduced glutathione was determined by the method of Ellman (1959). Total sulfhydryl groups were measured by the method of Ellman (1959). Vitamin C concentration was measured as previously reported (Omaye et al., 1979). Vitamin E ( $\alpha$ -tocopherol) was estimated by the method of Desai (1984). Superoxide dismutase activity was determined by the method of Kakkar et al. (1984). The activity of catalase was determined by the method of Sinha (1972). Glutathione peroxidase activity was estimated by the method of Rotruck et al. (1973). Glutathione S-transferase activity was determined by the method of Habig et al. (1974). Glutathione reductase was assayed by the method of Horn and Burns (1978). The estimation of glucose-6-phosphate dehydrogenase was carried out by the method of Beutler (1983). Total protein content of tissue homogenate was estimated as described previously (Lowry et al., 1951).

### Estimation of membrane-bound ATPase

The sediment after centrifugation was resuspended in ice-cold Tris-HCl buffer (0.1M) pH 7.4. This was used for the estimations of membrane-bound enzymes and protein content. The membrane bound enzymes such as Na<sup>+</sup>/K<sup>+</sup>-ATPase, Ca<sup>2+</sup>-ATPase and Mg<sup>2+</sup>-ATPase activity were assayed by estimating the amount of phosphorous liberated from the incubation mixture containing tissue homogenate, ATP and the respective chloride salt of the electrolytes (Bonting, 1970; Ohnishi et al., 1982; Hjerten and Pan, 1983). Total protein content was estimated by the method described by Lowry et al. (1951).

### Estimation of DNA damage by single-cell gel electrophoresis (Comet assay)

DNA damage was estimated by alkaline single-cell gel electrophoresis (Comet assay) according to the method of Singh et al. (1988). In this method, the cells were first lysed to form nucleoids. During electrophoresis, DNA fragments (from damaged DNA) streamed towards anode while the undamaged DNA trapped within the nucleus. When they are stained with SYBR green-I, damaged DNA gave the appearance of a comet tail and undamaged DNA gave spherical appearance. Prefrosted slides were prepared by pouring 3.0–5.0 mL of 1% normal agarose over clean glass slides. It was allowed to dry at room temperature and placed in hot-air oven at 70°C–80°C for 30 min. A freshly prepared suspension of 100 µL of hepatocytes in 1% low-melting point agarose (LMPA; 1:3 ratio) was cast on to prefrosted microscopic slides, immediately covered with cover slip and kept for 10 min in a refrigerator to solidify. Then the cover slip was removed and a top layer of 100 µL of LMPA was added and the slides were again cooled for 10 min. The cells were then lysed by immersing the slides in the lysis solution for 1 h at 4°C. After lysis, slides were placed in a horizontal electrophoresis tank. The unit was filled with electrophoresis buffer to a level of 0.25 cm above the slides. The cells were exposed to the alkaline electrophoresis solution for 20 min to allow DNA unwinding. Electrophoresis was conducted in a cold condition for 20 min at 25 V and 300 mA. After electrophoresis, the slides were placed horizontally and neutralized with Tris-HCl buffer. Finally, 50 µL of SYBR green-I (1:10,000) dilution) was added to each slide and analyzed using a fluorescence microscope. To prevent additional DNA damage, all steps were conducted under dimmed light or in the dark. Twenty five images were randomly selected from each sample and were examined at 200 magnification in a fluorescence microscope connected to a personal computer-based image analysis system, Komet v. 5.0 (Kinetics Imaging Ltd., Liverpool, UK). The relative amount of DNA appearing in the tail of the comet (percent tail DNA), tail length and tail moment (% tail DNA × length) were linearly related to DNA break frequency.

### Histopathological studies

The livers were removed and embedded in a paraffin block after being kept in Bouin fixative. Sections 4–5 mm thick were prepared from the blocks. These sections were stained with hematoxylin and eosin (H&E) to observe the general structure and viewed with the Olympus light microscope. To evaluate the degree of hepatic injury, 10 slides made from each treatment group were examined and assigned for severity of changes using scores on a scale of (–)=none, (+)=mild, (++)=moderate and (+++)=severe by two blinded histologists semi quantitatively. Sinusoidal dilatation, inflammatory cell infiltration, hepatocellular vacuolization and necrosis seen in all areas were identified as figures in Table 1. The histological changes were graded as follows: Sinusoidal dilatation:

Table 1. Histopathological scores in the liver of control and experimental rats.

Groups	Control	Arsenic +		
		Arsenic	Silibinin	Silibinin
Sinusoidal dilatation	–	+++	++	–
Inflammatory cell infiltration	–	+++	+	–
Hepatocellular vacuolization	–	+++	++	–
Necrosis	–	+++	+	–

Note: Pathological scores: (–)=none; (+)=mild; (++)=moderate; (+++)=severe.

(–)=normal sinusoids, (+)=mild dilatation, (++)=moderate dilatation, (+++)=severe dilatation; Cell infiltration: (–)=normal parenchyma and portal areas, (+)=mild infiltration especially in the periportal areas, (++)=moderate infiltration, (+++)=widespread infiltration; Hepatocellular vacuolization: (–)=normal hepatocytes, (+)=mild vacuolization, (++)=moderate vacuolization, (+++)=severe vacuolization; Hepatocellular necrosis: (–)=normal liver areas, (+)=mild necrosis, (++)=moderate necrosis, (+++)=widespread necrosis.

### Statistical analyses

Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using a commercially available statistics software package (SPSS® for Windows, V. 13.0, Chicago, USA). Results were presented as mean ± SD. *p* values < 0.05 were regarded as statistically significant.

### Results

Table 2 depicts the effect of arsenic and silibinin on food and water intake, body weight gain and organ-body weight ratio (%) in control and experimental rats. In arsenic treated rats, water and pellet diet consumption were significantly (*p*<0.05) decreased with decrease in body weight gain. A significant (*p*<0.05) increase in organ-body weight ratio was noted in arsenic treated rats. No significant changes were observed between control and silibinin treated rats. All these changes induced by arsenic intoxication were significantly (*p*<0.05) improved on oral administration of silibinin.

Figure 2 shows the levels of hepatic marker enzymes and bilirubin in control and experimental rats. Oral administration of arsenic caused abnormal liver function in rats. In arsenic-treated rats, the activities of serum hepatospecific enzymes, such as serum aminotransferase, alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase, gamma glutamyl transferase and the level of bilirubin were significantly (*p*<0.05) increased, when compared with control rats. But, oral administration of silibinin (75 mg/kg bw/day) to normal rats did not show any significant (*p*<0.05) effect on hepatic markers. Treatment of silibinin (75 mg/kg bw/day) with arsenic significantly (*p*<0.05) decreased the levels of hepatic markers and bilirubin.

Table 3 exhibits the levels of blood and hepatic ALAD in control and experimental animals. As intoxicated animals showed a significant ( $p < 0.05$ ) decrease in the levels of blood and hepatic ALAD when compared with control rats. Treatment with silibinin significantly ( $p < 0.05$ ) restored the altered levels of blood and hepatic ALAD by As to near normalcy levels in rats when compared with As-treated rats.

The changes in the levels of hepatic lipid peroxidation, hydroperoxides, protein carbonyl content and conjugated dienes in control and experimental rats are shown in Table 4. The levels of thiobarbituric acid reactive substances, lipid hydroperoxides, protein carbonyl content and conjugated dienes were significantly increased ( $p < 0.05$ ) in As-treated rats when compared with normal control rats. Oral administration of silibinin (75 mg/

Table 2. Changes in body weight, body weight gain, food intake, water intake and organ-body weight ratio in control and experimental rats.

Variables	Control	Arsenic	Arsenic + ilibinin	Silibinin
Body weight				
Initial (g)	191 ± 9.87	186 ± 9.24	188 ± 9.54	190 ± 9.68
Final (g)	232 ± 10.78	204 ± 10.52	221 ± 10.67	231 ± 10.72
Body weight gain (%)	21.35	9.67	17.55	21.57
Food intake (g/100 g bw/day)	12.08 ± 0.92 <sup>a</sup>	8.18 ± 0.67 <sup>b</sup>	10.55 ± 0.85 <sup>c</sup>	12.12 ± 0.87 <sup>a</sup>
Water intake (mL/rat/day)	18.40 ± 2.30 <sup>a</sup>	13.20 ± 1.20 <sup>b</sup>	16.30 ± 1.60 <sup>c</sup>	18.20 ± 2.10 <sup>a</sup>
Organ-body weight ratio (%) liver	2.92 ± 0.37 <sup>a</sup>	3.89 ± 0.51 <sup>b</sup>	3.27 ± 0.32 <sup>c</sup>	2.98 ± 0.34 <sup>a</sup>

Note: Values are expressed mean ± SD for six rats in each group; Values are not sharing a common superscript letter (a, b and c) differ significantly at  $p < 0.05$  (DMRT).

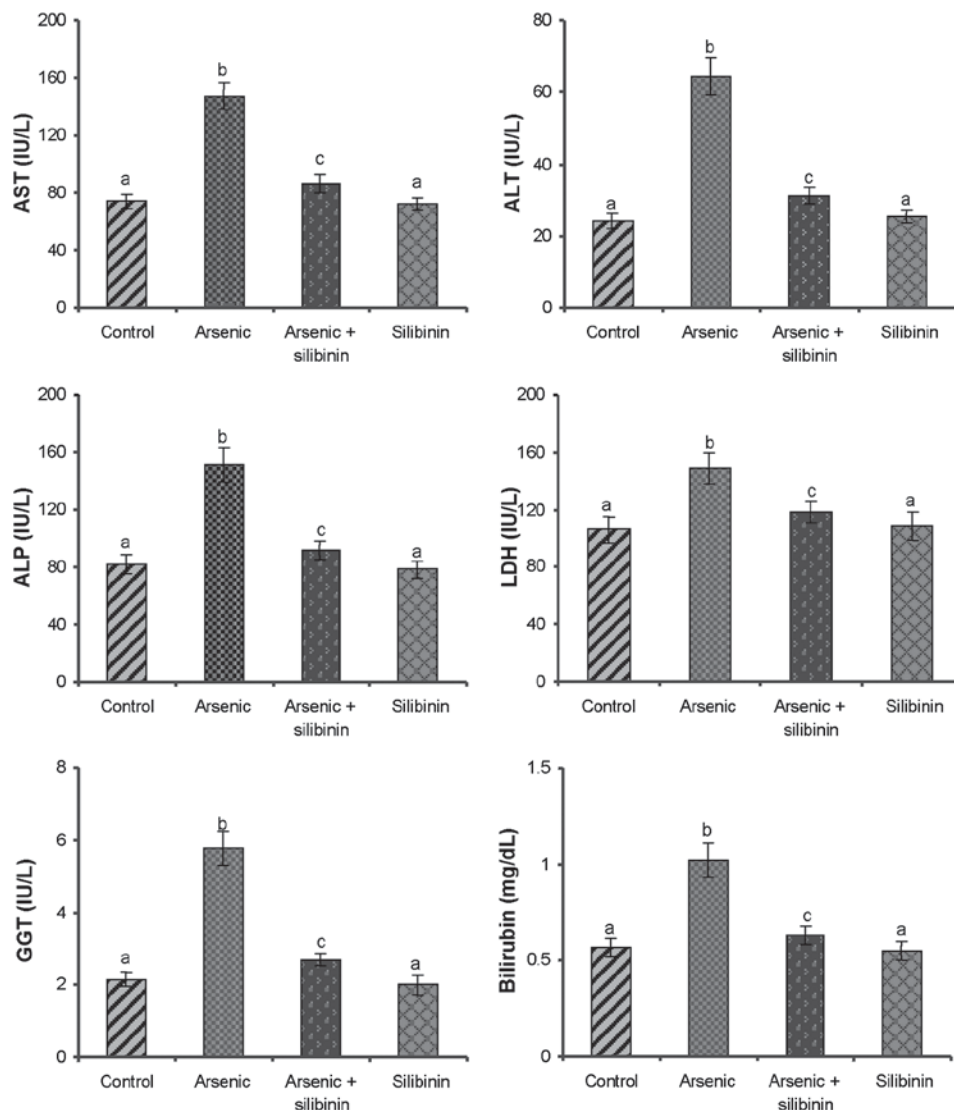


Figure 2. Effect of silibinin on the levels of serum hepatic enzyme markers of control and experimental rats. Values are mean ± SD for six rats in each group; Values are not sharing a common superscript letter (a, b and c) differ significantly at  $p < 0.05$  (DMRT).

kg bw/day) along with arsenic significantly lowered the levels of TBARS, LOOH, PCC and CD in the liver of rats when compared to As-treated rats.

Table 5 shows the changes in the levels of hepatic nonenzymatic antioxidants namely reduced glutathione, total sulfhydryl group, Vitamins C and E in the liver of control and experimental rats. A significant ( $p < 0.05$ ) decrease in the levels of hepatic nonenzymatic antioxidants was noticed in rats treated with As when compared to control rats. Treatment of silibinin (75 mg/kg bw/day) along with arsenic significantly

( $p < 0.05$ ) restored the levels of hepatic nonenzymatic antioxidants to near normal.

Table 6 illustrates the activities of enzymatic antioxidants namely superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, glutathione reductase and glucose-6-phosphate dehydrogenase in the liver of control and experimental rats. A significant ( $p < 0.05$ ) decrease in the activities of enzymatic antioxidants in As-treated rats was observed. Treatment of SB along with arsenic increased the levels of enzymatic antioxidants in the liver.

Table 3. Levels of blood and liver ALAD in control and experimental rats.

Groups	Control	Arsenic	Arsenic + Silibinin	Silibinin
Blood (nmol/min/mL)	9.27 ± 1.26 <sup>a</sup>	2.31 ± 0.34 <sup>b</sup>	5.64 ± 0.87 <sup>c</sup>	9.21 ± 1.32 <sup>a</sup>
Liver (nmol/min/mg protein)	38.41 ± 1.42 <sup>a</sup>	19.07 ± 0.97 <sup>b</sup>	27.69 ± 1.29 <sup>c</sup>	39.08 ± 1.34 <sup>a</sup>

Note: Values are mean ± SD for 6 rats in each group; Values are not sharing a common superscript letter (a, b and c) differ significantly at  $p < 0.05$  (DMRT).

Table 4. Effect of silibinin on the levels of TBARS, LOOH, CD and PC in the liver of control and experimental rats.

Groups	Control	Arsenic	Arsenic + Silibinin	Silibinin
TBARS (nmol/100g wet tissue)	0.73 ± 0.05 <sup>a</sup>	1.98 ± 0.13 <sup>b</sup>	0.92 ± 0.06 <sup>c</sup>	0.61 ± 0.05 <sup>d</sup>
LOOH (nmol/100g wet tissue)	91.76 ± 6.42 <sup>a</sup>	141.57 ± 10.79 <sup>b</sup>	108.29 ± 8.12 <sup>c</sup>	80.53 ± 7.89 <sup>d</sup>
CD (nmol/100g wet tissue)	67.94 ± 4.17 <sup>a</sup>	117.42 ± 8.79 <sup>b</sup>	78.34 ± 4.51 <sup>c</sup>	60.04 ± 3.47 <sup>d</sup>
PC (nmol/mg protein)	1.87 ± 0.19 <sup>a</sup>	4.58 ± 0.31 <sup>b</sup>	2.42 ± 0.23 <sup>c</sup>	1.69 ± 0.13 <sup>d</sup>

Note: Values are mean ± SD for 6 rats in each group; Values are not sharing a common superscript letter (a, b, c and d) differ significantly at  $p < 0.05$  (DMRT).

The levels of TBARS were expressed as nmol/100g wet tissue.

The levels of LOOH and CD were expressed as nmol/100g wet tissue.

The levels of PC were expressed as nmol/mg protein.

Table 5. Effect of silibinin on the levels of GSH, TSH, vitamins C and E in the liver of control and experimental rats.

Groups	Control	Arsenic	Arsenic + Silibinin	Silibinin
GSH (µg/mg protein)	14.28 ± 1.42 <sup>a</sup>	8.11 ± 0.67 <sup>b</sup>	12.01 ± 0.82 <sup>c</sup>	16.37 ± 1.54 <sup>d</sup>
TSH (µg/mg protein)	14.57 ± 0.25 <sup>a</sup>	8.71 ± 0.12 <sup>b</sup>	11.64 ± 0.21 <sup>c</sup>	15.72 ± 0.29 <sup>d</sup>
Vitamin C (µmol/mg tissue)	0.76 ± 0.08 <sup>a</sup>	0.44 ± 0.04 <sup>b</sup>	0.69 ± 0.07 <sup>c</sup>	0.83 ± 0.08 <sup>d</sup>
Vitamin E (µmol/mg tissue)	5.47 ± 0.52 <sup>a</sup>	3.23 ± 0.27 <sup>b</sup>	5.34 ± 0.38 <sup>c</sup>	5.76 ± 0.49 <sup>d</sup>

Note: Values are mean ± SD for 6 rats in each group; Values are not sharing a common superscript letter (a, b, c and d) differ significantly at  $p < 0.05$  (DMRT).

The levels of GSH and TSH were expressed as µg/mg protein.

The levels of vitamins C and E were expressed as µmol/mg tissue.

Table 6. Effect of silibinin on the levels of SOD, CAT, GPx, GST, GR and G6PD in the liver of control and experimental rats

Groups	Control	Arsenic	Arsenic + Silibinin	Silibinin
SOD (min/mg protein)	8.76 ± 0.71 <sup>a</sup>	4.27 ± 0.32 <sup>b</sup>	7.12 ± 0.54 <sup>c</sup>	9.16 ± 0.78 <sup>d</sup>
CAT (min/mg protein)	76.42 ± 6.57 <sup>a</sup>	54.38 ± 2.78 <sup>b</sup>	65.24 ± 6.24 <sup>c</sup>	81.32 ± 7.53 <sup>d</sup>
GPx (min/mg protein)	9.18 ± 0.59 <sup>a</sup>	5.48 ± 0.31 <sup>b</sup>	7.62 ± 0.67 <sup>c</sup>	9.37 ± 0.74 <sup>d</sup>
GST (min/mg protein)	19.7 ± 1.4 <sup>a</sup>	8.7 ± 1.1 <sup>b</sup>	16.8 ± 1.5 <sup>c</sup>	22.4 ± 1.7 <sup>d</sup>
GR (µg/mg protein)	0.34 ± 0.06 <sup>a</sup>	0.17 ± 0.02 <sup>b</sup>	0.27 ± 0.04 <sup>c</sup>	0.37 ± 0.06 <sup>d</sup>
G6PD (µg/mg protein)	2.47 ± 0.24 <sup>a</sup>	1.76 ± 0.17 <sup>b</sup>	2.24 ± 0.21 <sup>c</sup>	2.52 ± 0.28 <sup>d</sup>

Note: Values are mean ± SD for 6 rats in each group; Values are not sharing a common superscript letter (a, b, c and d) differ significantly at  $p < 0.05$  (DMRT).

SOD—One unit of enzyme activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in one minute/mg protein.

CAT - µmol of H<sub>2</sub>O<sub>2</sub> utilized/min/mg protein.

GPx - µg of GSH consumed/min/mg protein.

GST - µmol of CDNB-GSH conjugate formed/min/mg protein.

The activities of GR were expressed as nmol of NADPH oxidized/min/mg protein.

G6PD were expressed as nmol of NADPH formed/min/mg protein.

Table 7 shows the activities of hepatic  $\text{Na}^+/\text{K}^+$ ,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ATPases in the liver of control and experimental rats. A significant ( $p < 0.05$ ) decrease in the activities of membrane bound ATPases in the liver tissue of As-treated rats when compared with control rats. Treatment of silibinin (75 mg/kg bw/day) along with arsenic significantly ( $p < 0.05$ ) restored the levels of membrane bound ATPases in the liver of arsenic intoxicated rats. No significant changes were observed between control and silibinin treated rats in the activities of these membrane bound ATPases.

Figure 3 shows the alterations in the comet assay parameters of control and experimental rats. A significant ( $p < 0.05$ ) increase in different comet assay parameter such as % DNA in tail, tail length and tail movement was observed in rats treated with As when compared with control rats. Treatment SB along with As significantly ( $p < 0.05$ ) reduced the % DNA in tail, tail length and tail movement in hepatocytes. Control and SB alone treated rats showed no or minimal DNA migration.

Histopathological studies showed that arsenic administration induces the severe pathological changes in the liver. The liver of control rats (Figure 4A) and silibinin- (Figure 4E) treated rats showed a normal histoarchitectural pattern of liver. Arsenic exposure resulted in extensive degeneration of hepatocytes with necrosis, inflammation, vacuolization, inflammatory cell infiltration and sinusoidal dilation (Figures 4B and C). Arsenic along with silibinin administration (Figure 4D) showed near normal hepatocytes without any pathological signs.

## Discussion

The findings of the present study clearly revealed the protective nature of silibinin against arsenic-induced oxidative stress mediated hepatotoxicity in rats. Body weight reduction is used as a crucial indicator for the disintegration of general health status of an organism. In the present investigation, administration of arsenic at a dose of 5 mg/kg bw induced hepatic toxicity as evidenced by the biochemical and pathological alterations with no mortality. It has been reported that arsenic accumulation causes disturbances in the total body weight, absolute and relative liver weight of rats may be due to regenerative changes of arsenic in hepatic cells (Yousef et al., 2008; El-Demerdash et al., 2009). Our results are coincided with these reports as

arsenic exposed rats showed decreased intake of water and food accompanied with retardation in growth rate and alterations in liver-body weight ratio. All these morphological changes observed in arsenic intoxi-

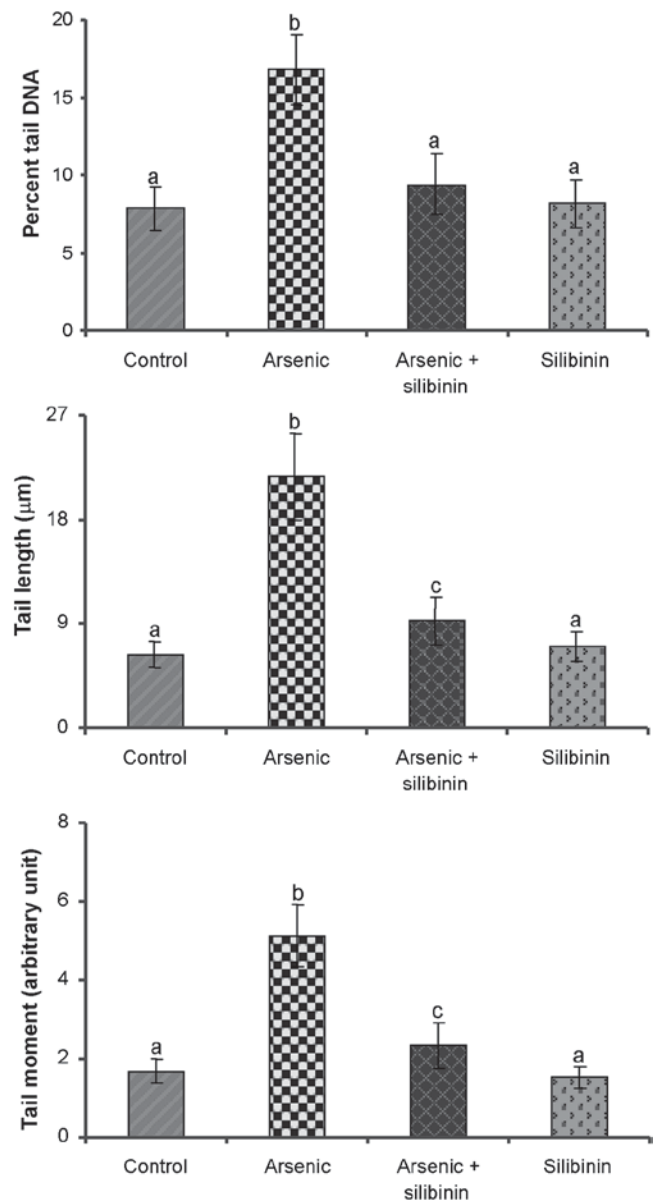


Figure 3. Effect of silibinin on DNA damage in the hepatocytes of control and experimental rats. Arbitrary unit = Percentage tail DNA  $\times$  tail length; Values are mean  $\pm$  SD for six rats in each group; Values are not sharing a common superscript letter (a, b and c) differ significantly at  $p < 0.05$  (DMRT).

Table 7. Levels of membrane bound adenosine triphosphatases (ATPase) enzymes in the liver of control and experimental rats.

Groups	Control	Arsenic	Arsenic +Silibinin	Silibinin
Total ATPases	1.92 $\pm$ 0.16 <sup>a</sup>	1.24 $\pm$ 0.13 <sup>b</sup>	1.79 $\pm$ 0.15 <sup>c</sup>	1.95 $\pm$ 0.18 <sup>a</sup>
$\text{Na}^+\text{K}^+$ ATPases	0.43 $\pm$ 0.04 <sup>a</sup>	0.23 $\pm$ 0.02 <sup>b</sup>	0.37 $\pm$ 0.03 <sup>c</sup>	0.44 $\pm$ 0.04 <sup>a</sup>
$\text{Ca}^{2+}$ ATPases	0.57 $\pm$ 0.07 <sup>a</sup>	0.32 $\pm$ 0.03 <sup>b</sup>	0.51 $\pm$ 0.06 <sup>c</sup>	0.58 $\pm$ 0.07 <sup>a</sup>
$\text{Mg}^{2+}$ ATPases	0.73 $\pm$ 0.08 <sup>a</sup>	0.54 $\pm$ 0.05 <sup>b</sup>	0.65 $\pm$ 0.06 <sup>c</sup>	0.74 $\pm$ 0.07 <sup>a</sup>

Note: Values are mean  $\pm$  SD for six rats in each group; Values are not sharing a common superscript letter (a, b, and c) differ significantly at  $p < 0.05$  (DMRT); Units for ATPases  $\mu\text{g pi liberated}/\text{min}/\text{mg protein}$ .

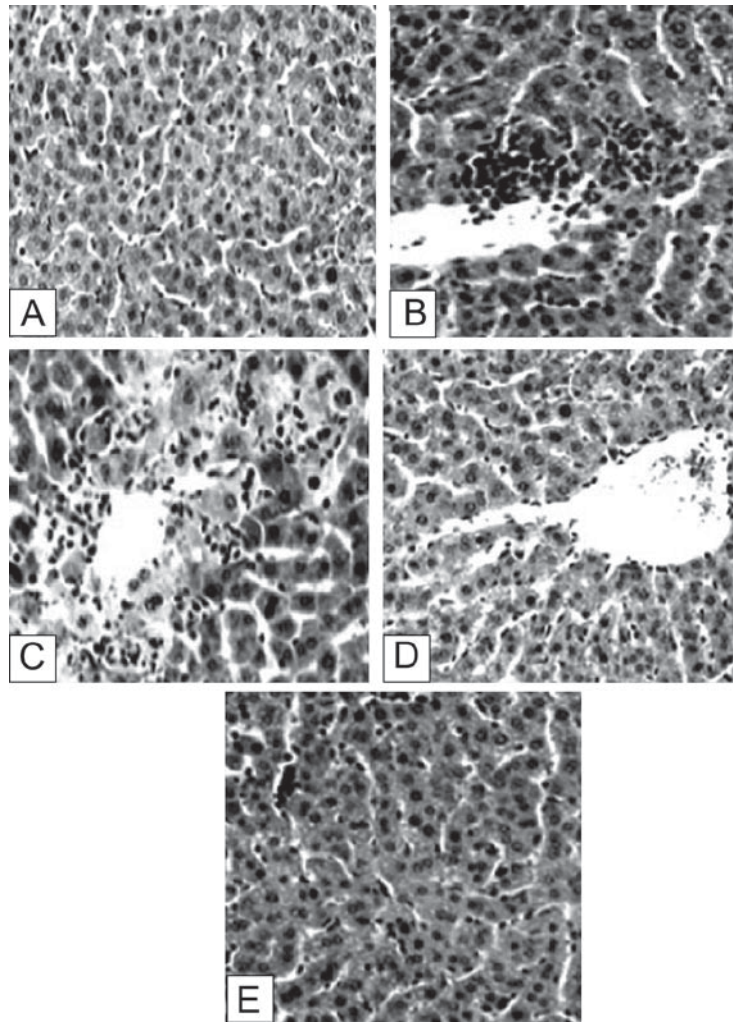


Figure 4. Representative photographs from the liver of control and experimental rats: (A) Section of control liver showing normal arrangement of sinusoids and hepatocytes (H and E, 100 $\times$ ). (B) Section of liver treated with arsenic showing the extensive inflammation, dilated sinusoids, degeneration of hepatocytes with necrosis, vacuolization and inflammatory cell infiltration. (H and E, 100 $\times$ ). (C) Section of liver treated with arsenic showing severe necrotic changes, severe inflammation, fatty degeneration of hepatocytes and complete derangement of hepatic cords (H and E, 100 $\times$ ). (D) Section of liver treated with arsenic and silibinin showing significant improvement of hepatic histoarchitecture with mild infiltration (H and E, 100 $\times$ ). (E) Section of liver treated with silibinin alone showing the normal histoarchitectural pattern of hepatic parenchyma (H and E, 100 $\times$ ).

cated rats were potentially ameliorated by treatment with silibinin.

Arsenic is known to cause hepatic tissue injury as reflected from a significant increase in serum hepatic marker enzymes indicating the cellular leakage and loss of functional integrity of hepatic membrane architecture (Gaskill et al., 2005). In the present study, arsenic intoxication caused a significant increase in the activities of AST, ALT, ALP and LDH, probably resulting from hepatocyte membrane damage. GGT is known as an important liver function marker and has been used as an index of liver dysfunction. Recent studies reported that measurement of serum GGT might be useful in studying oxidative stress-related tissues. The products of the GGT reaction may themselves lead to increased free-radical production, particularly in the presence of iron (Lee et al., 2004). Previous studies showed that increase in plasma total bilirubin concentrations was associated

with free radical production in rats treated with arsenic (Nandi et al., 2005). The elevated levels of serum hepatic marker enzymes and bilirubin of the rats exposed to As indicate loss of functional integrity of hepatic membranes and their increased permeability leading to leakage of hepatic enzymes into the blood stream and abnormal liver function (Banerjee et al., 2009). Administration of silibinin ameliorated arsenic-induced hepatotoxicity as shown by the decreased levels of AST, ALT, ALP, LDH and GGT and reduced level of serum bilirubin thus offering protection against arsenic toxicity by stabilizing the cell membrane of rat hepatocytes. Administration of silibinin at a dose of 75 mg/kg body weight more remarkably improved the alterations in hepatic function indicator induced by arsenic. silibinin has already been reported to exhibit membrane-stabilization properties against ROS-mediated oxidative hepatocellular injury (Basiglio et al., 2009).



$\delta$ -Aminolevulinic acid dehydratase (ALAD) is a sulfhydryl containing enzyme that catalyzes the asymmetric condensation of two molecules of aminolevulinic acid (ALA-substrate) to prophobilinogen during heme synthesis pathway. Administration of arsenic exerted a significant depletion in the levels of blood and liver ALAD. Since arsenic has a high affinity for sulfhydryl, this may lead to possible inhibition of ALAD activity. An inhibition in the activity of blood and liver ALAD may lead to a significant accumulation of ALA. Therefore, there was a possibility of increased ALA might generate more reactive oxygen intermediates (ROIs) and thus oxidative stress (Bhadoria and Flora, 2003). Restoration of ALAD activity has been observed in arsenic along with silibinin-treated rats. This beneficial effect may be due to the direct scavenging effects of reactive oxygen species by silibinin and its ability to inhibit thiol group oxidation (Haddad et al., 2009).

Arsenic acts as a pro-oxidant in biological systems and causes lipid peroxidation which is a basic cellular deteriorating process in the liver (Flora, 1999). Profound free-radical generation and enhanced lipid peroxidation are the dual facets of oxidative stress that initiate the pathogenesis of arsenic-induced hepatotoxicity (Bashir et al., 2006). In addition to lipid peroxidation, protein carbonylation also served as a validated marker for protein oxidation particularly for the proteins containing amino acid residues like lysine, arginine, proline, threonine and glutamic acid. In the present investigation, there was a significant increase in the level of TBARS, LOOH, PCC and CD in the liver of arsenic-exposed rats which confirms the onset of hepatic oxidative stress. Decreased levels of these oxidative stress markers in the liver of arsenic-treated rats administered with silibinin revealed the radical scavenging activity of silibinin acid which could be due to the presence of one methoxy group and three hydroxyl groups at 3rd, 5th and 7th positions, respectively (Rajnarayana et al., 2001).

Thiol-based antioxidant system contributes a second line of cellular defense against reactive free radicals and other oxidant species-mediated oxidative damage. In the present study, there was a significant decrease in the level of GSH, TSH, Vitamins C and E in the liver of arsenic exposed rats which fortifies the onset of hepatic oxidative stress. Several pathways have been proposed to elucidate the depletion in arsenic-induced cellular GSH levels. Thiols are thought to play a crucial role in protecting cells against reactive oxygen species. Depleted levels of TSH and GSH represent an excess free radical production, which may be due to the binding of arsenic with various sulfhydryls that exist in the cell (Kokilavani et al., 2005). The nonenzymatic antioxidants GSH, vitamin C and vitamin E are interrelated by a recycling process. 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 GSH (George,

2003). Restoration of these antioxidants in the liver of arsenic along with silibinin could be related to its free-radical scavenging activity (Ligeret et al., 2008). Increase in GSH levels in turn contributes to the recycling of other antioxidants such as vitamins C and E (Exner et al., 2000). Therefore, this property of silibinin might have resulted in the recoupment of the activities of the nonenzymatic antioxidants to near normalcy.

Antioxidant enzymes, such as SOD, CAT, GST, GR and GPx are considered to be the first line of cellular defense against oxidative injury. Status of these antioxidant enzymes is an appropriate indirect way to assess the prooxidant-antioxidant status in arsenic induced toxicity. Among them SOD and CAT mutually function as important enzymes in the elimination of ROS and RNS. SOD is an antioxidant enzyme which catalyzes the dismutation of superoxide to H<sub>2</sub>O<sub>2</sub> which in turn is removed by CAT (Usuh et al., 2005). Thus, SOD can act as a primary defense against superoxide anion and prevents further generation of free radicals. Reduction in SOD activity in arsenic-exposed animals reflects enhanced production of superoxide radical anions (Yamanaka et al., 1991). The increase in superoxide radicals also inhibits catalase activity (Kono, 1982). NADPH is required for the activation of CAT from its inactivated form. Thus, reduced activity of catalase in arsenic exposed animals may be due to the insufficient supply of NADPH during arsenic metabolism (Kirkman and Gaetani, 1984). On the other hand, glutathione-related enzymes, such as GPx, GR and GST function either directly or indirectly as antioxidants. GST is a family of proteins involved in the detoxification process by catalyzing the reaction of glutathione with toxicants to form an S-substituted glutathione (Danyelle and Kenneth, 2003). GPx is a selenium-containing enzyme, it is well established that arsenic interacts with essential seleno-cysteine moiety of the enzyme to form insoluble and inactive arsenic-selenium complex (Flora et al., 1999) rendering it unavailable and ultimately resulting in the inhibition of GPx activity or altering the expression and synthesis of selenoproteins like GPx (Ganyc et al., 2007). GST and GPx play principle function to reduce organic hydroperoxides within membranes and lipoproteins in the presence of GSH. Therefore, decreased activities of GST and GPx with a concomitant decrease in the activity of GSH-regenerating enzyme, GR suggest the consumption of glutathione while protecting against the arsenic-induced oxidative stress, as they help to maintain cellular redox status. G6PD is an important enzyme of hexosemonophosphate (HMP) shunt. It converts one molecule of glucose-6-phosphate into 6-phosphogluconolactone in the presence of Mg<sup>2+</sup>, Mn<sup>2+</sup> and Ca<sup>2+</sup> ions and subsequently NADP<sup>+</sup> is reduced to NADPH. A subsequent reduction of the G6PD activity in arsenic-induced rats showed impaired generation of NADPH which is required for the reduction of GSSG to GSH (Hughes, 2002; Shila et al., 2005). In the present study, it has been observed that arsenic

intoxicated rats significantly reduced the activities of all the antioxidant enzymes. Interestingly, the fact that silibinin could markedly renew the impairment of antioxidant defense system in the liver of arsenic-treated rats might be attributed to its antioxidant and chelating properties which could be due to the orientation of hydroxyl groups in the 3rd, 5th and 7th positions in silibinin (Rajnarayana et al., 2001).

The determination of membrane-associated enzyme activities like adenosine triphosphatases (ATPases) indicates the changes in membranes under pathological conditions (Kempaiah and Srinivasan, 2006). ATPases are lipid-dependent membrane-bound enzymes, which play a central role in the active transport of ions, maintenance of cellular homeostasis and are also involved in neurotransmission process, maintenance of ion gradients and regulation of cell volume. The peroxidation of membrane lipids not only alters the structural as well as functional integrity of cell membranes, but also affects the activities of various membrane-bound enzymes including  $Mg^{2+}$  ATPase,  $Ca^{2+}$  ATPase and  $Na^+/K^+$  ATPase, which in turn lead to disruption in cellular homeostasis (Hazarika et al., 2001).

In the present study, a significant decrease in the activities of membrane-bound total ATPases in the liver was observed in arsenic-treated rats. Decreased activity of  $Na^+/K^+$  ATPase could be due to enhanced lipid peroxidation by free radicals on As induction, since  $Na^+/K^+$  ATPase is a "SH" group containing enzyme and is lipid dependent (Ithayarasi and Devi, 1997). Decreased activity of  $Na^+/K^+$  ATPase can lead to a decrease in sodium efflux, thereby altering membrane permeability (Finotti and Palatine, 1986). The disruption of membrane permeability or fragmentation of the membrane leads to the leakage of  $Ca^{2+}$  ions into cells thereby potentiating irreversible cell destruction. The  $Ca^{2+}$  overload mediated arsenic also decreased the  $Ca^{2+}$  ATPase activity in cell membrane. It is generally accepted that due to high affinity for SH groups, arsenic binds avidly to various enzyme proteins and inactivates them.  $Mg^{2+}$  ATPase activity is involved in other energy requiring process in the cell and its activity is sensitive to lipid peroxidation. Administration of silibinin in arsenic intoxicated rats significantly reduced the lipid peroxidation in liver tissue and sustained the activities of membrane bound enzymes. This could be due to the ability of silibinin to protect the SH groups from the oxidative damage through the inhibition of peroxidation of membrane lipids and stabilizes the membrane (Basiglio et al., 2009).

The comet assay is a rapid, sensitive and versatile method for the quantification of DNA damage in the individual cells (Fairbairn et al., 1995). The levels of % DNA in tail, tail length, and tail movement significantly increased in the hepatocytes of arsenic-treated rats when compared with control group. Previous reports show that lipid peroxidation products of polyunsaturated fatty acids play a major role in genotoxicity of the

cell (Comporti, 1989). Several mutagenic and genotoxic lipid peroxidation products, in particular, malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) have been identified to bind to DNA and to damage it (Eder et al., 2006). This might be the main reason for increased severity of DNA damage in arsenic-treated cells. Our findings are consistent with the other published reports, which show that arsenic produces DNA strand breaks and chromosomal aberrations in treated cells (Kligerman et al., 2010). DNA damage significantly reduced in the hepatocytes of silibinin treated rats. Silibinin is a well-known free-radical scavenger and thereby it reduces the ROS mediated lipid peroxidation and oxidative DNA damage by arsenic. Daille et al. (2008) demonstrated that silibinin acts as an effective scavenger of reactive oxygen radicals in perfused hepatocytes and thereby reduced the oxidative stress. Thus, silibinin, by virtue of its free-radical scavenging capacity, antiinflammatory property and the ability to replenish the glutathione, might have reduced cellular and DNA damage caused by arsenic.

In the present study, biochemical and molecular perturbations induced by arsenic were well correlated with the histopathological findings. Arsenic intoxication caused severe pathological changes such as degeneration of hepatocytes, inflammatory cell infiltration, dilated sinusoids and focal necrosis in liver. Administration of silibinin attenuated most of the histopathological changes induced by arsenic probably through its capacity to scavenge lipid peroxy radicals before they attack membrane lipids and thereby maintains the normal hepatic histoarchitecture (Erlejman et al., 2004).

In conclusion, the current data supports that silibinin acts as a potential cytoprotective agent, which might prevent the arsenic induced oxidative hepatotoxicity by decreasing lipid peroxidation, protein oxidation, DNA damage and augmenting the enzymatic and nonenzymatic antioxidant status in liver. The present investigation also provides a well-validated supportive literature about counteracting the arsenic toxicity with silibinin in arsenic prone zone. However, further investigations are noteworthy to elucidate the precise molecular mechanism of silibinin protection against arsenic-induced cytotoxicity.

## Declaration of interest

The authors report no declarations of interest.

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