

Silibinin attenuates arsenic induced alterations in serum and hepatic lipid profiles in rats

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ABSTRACT

To study the protective effect of silibinin against arsenic induced alterations in the serum and hepatic lipid profiles in rats. In this context, the chronic oral exposure of sodium arsenite as the source of arsenic (As) at 5mg/kg/bw with silibinin (SB) 75mg/kg/bw for 28 days. In the present experiment, the elevated levels of serum hepatic markers such as aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and serum cholesterol, triglycerides, low density lipoprotein (LDL), very Low Density Lipoprotein (VLDL), tissue triglycerides and cholesterol with concomitant decrease in serum HDL and tissue phospholipids were observed in As intoxicated rats. Pre-administration of SB with As was found to protect against adverse changes in the serum and hepatic tissue lipid profile in rats. Our results suggest that SB exerts a hypolipidemic effect against arsenic induced toxicity in liver by restoring the altered levels of lipids. The protective effects of SB on serum and liver as evidenced by the clear restoration of lipid profile in rats.

INTRODUCTION

Arsenic, the naturally occurring metalloid, exerts its carcinogenic and genotoxic effect on living organism in different parts of the world (Hartwig *et al.*, 1997). And also the derivative of arsenic as herbicides; insecticides, rodenticides, food preservatives, and fossil fuel are drastically contaminating drinking water (Bode and Dong, 2002; Yih *et al.*, 2002). Moreover, As induced toxicity has been reported to be associated with a variety of cancers, dermatitis, cardiovascular diseases, peripheral neuropathy, diabetes mellitus, (Wang *et al.*, 2002; Chen *et al.*, 2007; Mumford *et al.*, 2007; Tapio and Grosche, 2006; Meliker *et al.*, 2007; Mazumder *et al.*, 1998) renal failure and liver dysfunction (Miltonprabu and Muthumani, 2012; Muthumani and Miltonprabu, 2012a). Therefore, liver disease could be a major risk of environmental arsenic exposure in World wide. The liver is the primary target organ for the metabolism of arsenicals.

The major metabolic pathway of inorganic arsenic in humans is its methylation in the liver. Arsenite salt may exert its toxicity through reactions with thiols in cells (Hossain *et al.*, 2000; Akhand *et al.*, 2002) especially vicinal dithiols (Flora *et al.*, 2008). On the other hand, recent results suggest that arsenic may also exert its toxicity through the generation of reactive oxygen species (ROS). Arsenic compounds during their metabolism in cells may generate reactive oxygen species (Muthumani and Miltonprabu, 2012b). In addition, oxidative DNA damage, acquired tolerance to apoptosis, enhanced cell proliferation, altered DNA methylation, genomic instability, and aberrant estrogens signalling have been reported to be involved in the liver toxicity caused by arsenic (Rossman, 2003). Hepatic cancer and other hepatic disorders are considered to be the major causes of arsenic-related mortality (Liu *et al.*, 1992; Liu *et al.*, 2002; Zhou *et al.*, 2002). Earlier literatures showed that the plant constituents with antihepatotoxic potential, a number of medicinal preparations proved to exhibit a protective effect against As induced organ toxicity (Muthumani and Miltonprabu, 2012b).

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Silibinin (SB), a polyphenolic flavonoid antioxidant of silymarin, isolated from the seeds or fruits of milk thistle (*Silbum marianum* (L.) Gaertn.), has been used as a traditional medicine, against various hepatic ailments (Saller *et al.*, 2001) and cancer (Singh and Agawal,2002).SB is reported to have a broad spectrum of biological activities such as hepatoprotective (Ferenci,1989), antioxidant (Saller *et al.*, 2001), metal chelation (Pietrangelo *et al.*, 1995) free radical-scavenging (Winterbourn,2008). The present study was carried out to investigate the effect of SB on hepatic toxicity assessed by serum and hepatic tissue lipid profiles in rats intoxicated by As.

MATERIALS AND METHODS

Chemicals

Arsenic, silibinin, 1,1',3,3'-tetramethoxy propane, bovine serum albumin, Hank's 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. Chemical structure of silibinin is shown in Fig. 1.

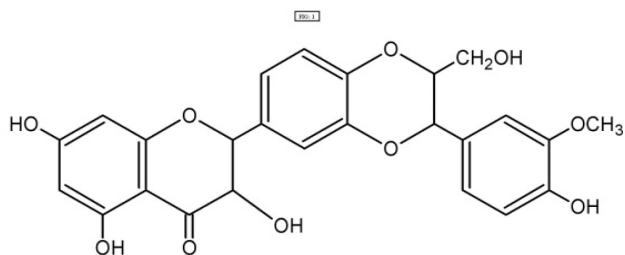


Fig. 1: Chemical structure of silibinin (C₂₅H₂₁O₁₀).

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 in order 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 semi-natural light/dark condition (12 h light/12 h 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, As was administered intragastrically at a dose of 5 mg/kg body weight/ day for 4 weeks, which was 1/8 of the oral LD₅₀ 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.

Group 1: Rats were administered with normal saline and CMC solution for 4 weeks and treated as control.

Group 2: Rats were administered with As (5 mg/kg BW day) for 4 weeks and treated as toxic control.

Group 3: Rats were pre-administered with SB, (75 mg/kg BW day) followed by As (5 mg/kg BW day) for 4 weeks.

Group 4: Rats were administered with SB alone (75 mg/kg BW day) 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 anaesthetised with an intramuscular injection of ketamine hydrochloride (24 mg/kg) and sacrificed by decapitation. Blood was collected and serum was separated and used for lipid analysis. The liver tissue was dissected out, washed in ice-cold saline, and patted dry and weighed. The liver tissue was used for the lipid extraction.

Activities of serum marker enzymes

The activities of serum aspartate aminotransferase (E.C. 2.6.1.1), alanine aminotransferase (E.C. 2.6.1.2) and alkaline phosphatase (E.C.3.1.3.1) were assayed using commercially available diagnostic kits (Sigma diagnostics (I) Pvt. Ltd., Baroda, India).

Lipid extraction from liver tissue

To 500mg of the liver tissue 150mL of chloroform methanol mixture in the ratio (2: 1) was added and the homogenate was prepared. This step was repeated three times to completely homogenize the residue and extract the lipid. The three extracts were pooled and the volume was measured. The contents were transferred to a separating funnel. The chloroform layer was then transferred to a flat bottom flask through anhydrous Na₂SO₄. The contents were flash evaporated to concentrate the extract. This concentrated mixture was then made to a known volume with chloroform. A volume of 1mL of the extract was transferred to a preweighed vial and aliquots were taken for the following estimations.

Lipid estimation

Cholesterol was estimated by the method of Parekh and Jung (1970). Phospholipids were estimated according to the method of Rouser *et al* (1970). Triglycerides were estimated according to the method of Rice (1970).

Serum lipoproteins

Serum Lipoproteins were fractionated by a dual precipitation technique as described by Wilson *et al* (1988).

Histopathological studies

For qualitative analysis of liver histology, the tissue samples were fixed for 48 h in 10% formalin-saline and dehydrated by passing successfully in different mixture of ethyl alcohol, water, cleaned in xylene and embedded in paraffin. Sections of the tissues (5-6 μ m thick) were prepared by using a rotary microtome and stained with haematoxylin and eosin dye, which was mounted in a neutral deparaffined xylene medium for microscopical observations. Six rats from each group were sacrificed for analyzing the hepatic histological examinations.

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 \pm SD. P values <0.05 were regarded as statistically significant.

RESULTS

General effect of SB in As-treated rats

Table: 1 represents the effect of As and SB on food and water intake, body weight gain and liver-body weight ratio (%) in control and experimental animals. In As treated rats, water and pellet diet consumption significantly ($P<0.05$) reduced with decrease in body weight gain. A significant ($P<0.05$) increase in liver-body weight ratio was noticed in As+SB treated. No significant changes were observed between control and SB administered rats.

Serum hepatic Markers

The serum hepatic marker activities of ALT, AST, and ALP were significantly ($P<0.05$) increased in the arsenic-treated rats when compared to the normal control group (Fig. 2). Pretreatment with SB leads to the protective role in the restoration of these hepatic markers was observed in the As treated with SB group.

Serum total cholesterol triglycerides, HDL, LDL and VLDL levels

Serum total cholesterol, triglyceride LDL and VLDL levels were significantly increased ($P<0.05$) and that of HDL level was decreased significantly ($P<0.05$) in As administered rats compared to that of control animals. In rats pretreated with SB along with As the HDL level was significantly increased ($P<0.05$), whereas serum cholesterol, triglyceride, LDL and VLDL levels were significantly reduced ($P<0.001$) compared to rats treated with As alone. These levels were no significant in rats treated with SB alone when compared to the control (Fig 3).

Tissue total cholesterol, phospholipid and triglyceride levels

The tissue phospholipid decreased significantly ($P<0.05$) whereas tissue total cholesterol and triglyceride levels were found to be significantly increased ($P<0.05$) in rats administered As alone.

Rats pretreated with SB along with As registered a significant increase ($P<0.05$) in tissue phospholipid content whereas tissue cholesterol and triglyceride levels were decreased significantly ($P<0.05$) compared to rats treated with As alone. These levels in SB alone treated rats were no significant when compared to control animals (Fig 4).

Histopathological Studies

Histopathological investigations showed that the administrations of As in rats produced severe hepatic damage including extensive degeneration of hepatocytes with necrosis, inflammation, vacuolization, inflammatory cell infiltration and fatty degenerative changes (Figs. 5B) when compared with control rats (Fig. 5A).

The histopathological abnormalities induced by As were significantly attenuated in the liver of rats administered with SB (Fig. 5C). The histoarchitectural pattern of liver was almost normal in SB administered rats (Fig. 5D).

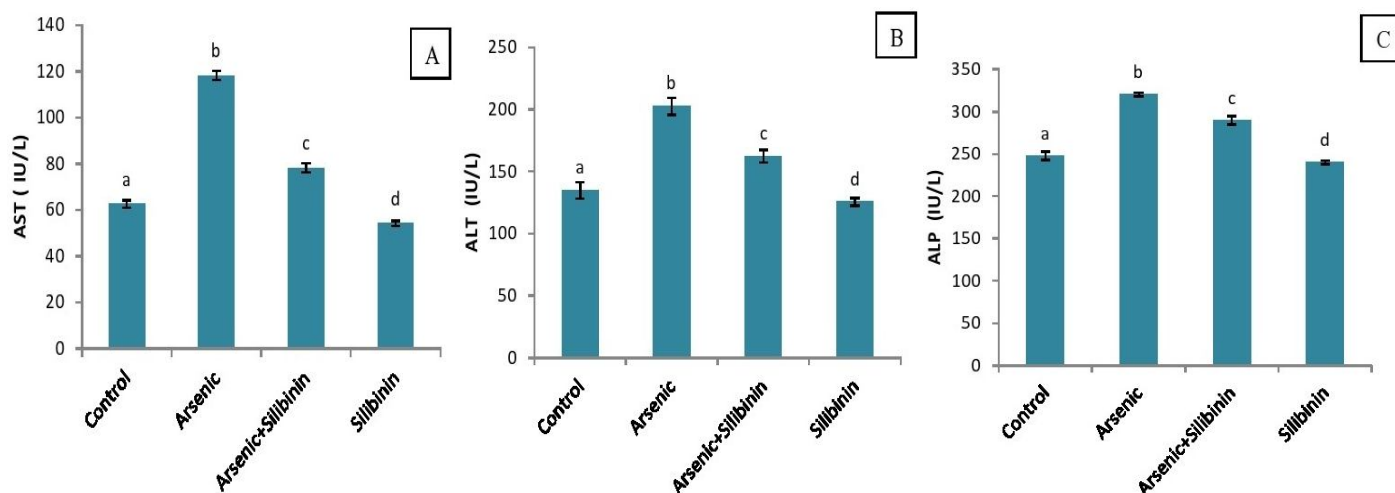


Fig. 2: The effect of As and SB supplementation on serum hepatic enzyme markers such as AST (A), ALT (B) and ALP (C) of control and experimental rats. Values are mean \pm SD for 6 rats in each group; ^{a, b, c and d} Values are not sharing a common superscript letter (a, b and c) differ significantly at $P<0.05$ (DMRT).

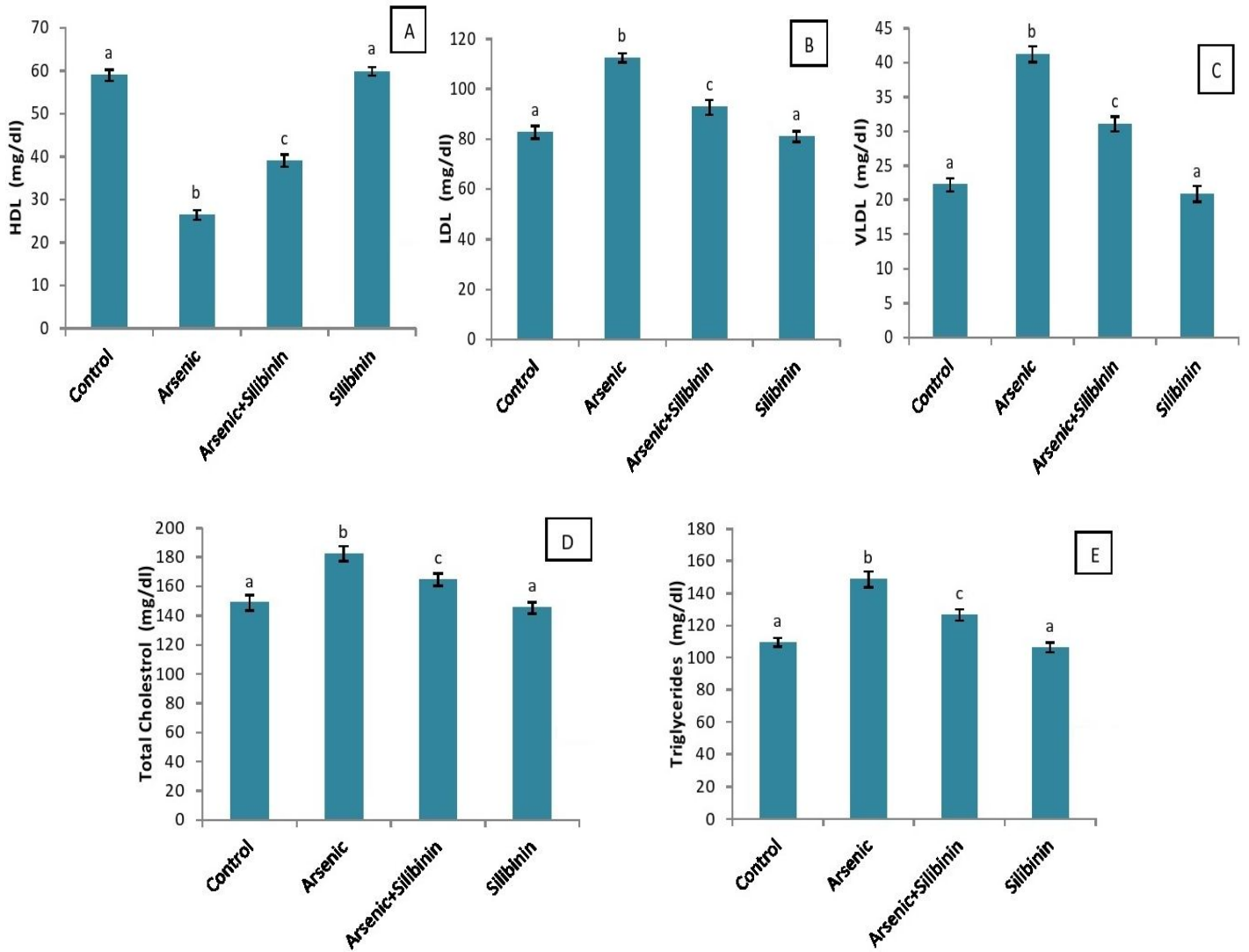


Fig. 3: The effects of As and SB supplementation on serum HDL (A), LDL (B), VLDL (C), total cholesterol (D) and triglyceride (E) levels. Values are mean±SD for 6 rats in each group; ^{a, b and c}Values are not sharing a common superscript letter (a, b and c) differ significantly at P<0.05 (DMRT).

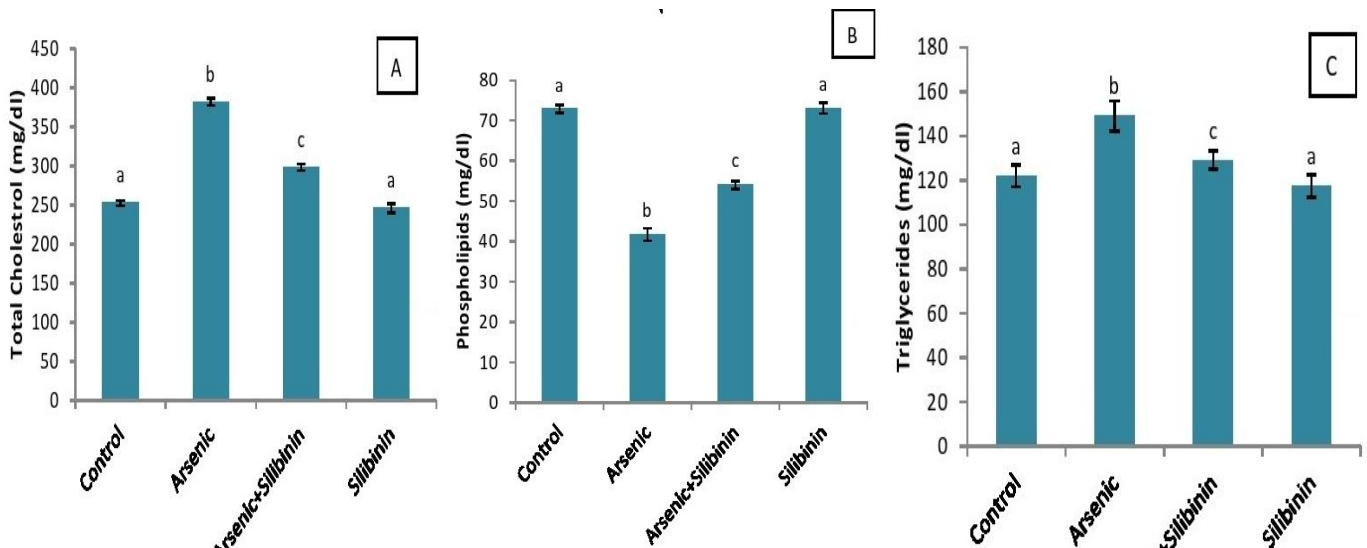


Fig. 4: The effects of As and SB supplementation on tissue total cholesterol (A), phospholipid (B) and triglyceride (C) levels. Values are mean±SD for 6 rats in each group; ^{a, b and c}Values are not sharing a common superscript letter (a, b and c) differ significantly at P<0.05 (DMRT).

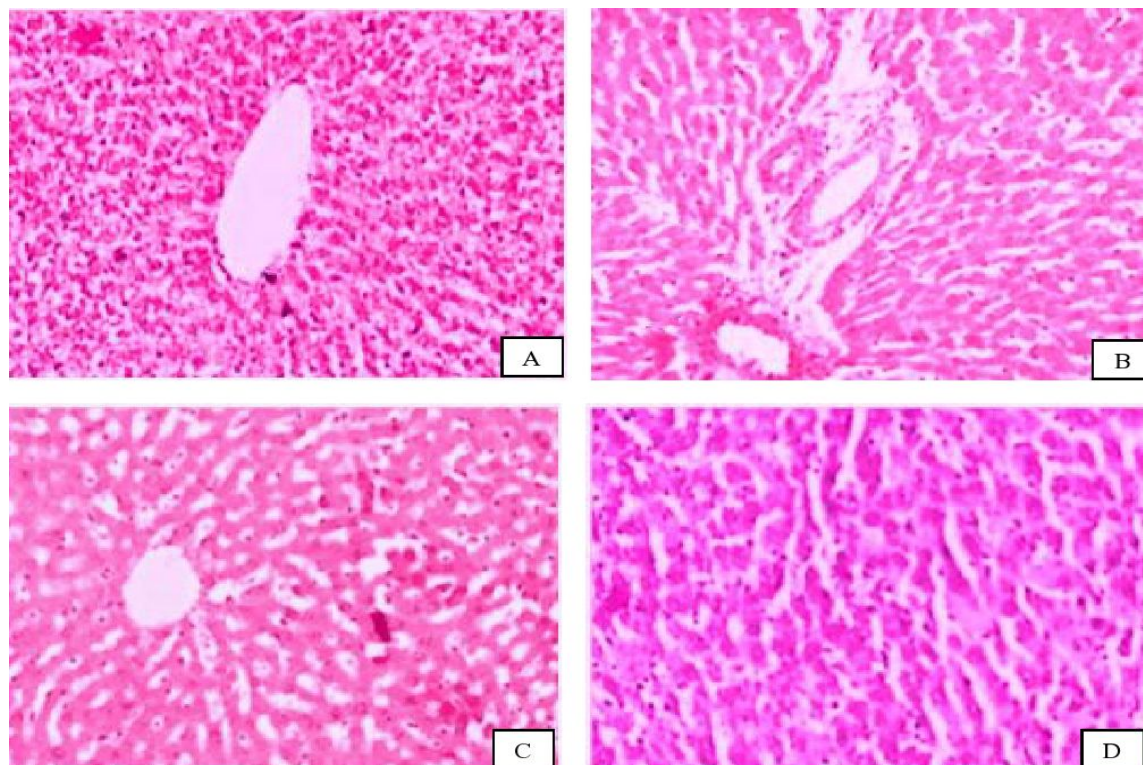


Fig. 5: Representative photographs from the liver of control and experimental rats: A) section of control liver showing the central vein and normal arrangement of hepatocytes around the central vein (H and E, 40X), B) section of liver treated with arsenic showing the extensive inflammation, degeneration of hepatocytes with necrosis, vacuolization, inflammatory cell infiltration and fatty degenerative changes (H and E, 40X), C) section of liver treated with arsenic and silibinin showing significant improvement of hepatic histoarchitecture (H and E, 40X) and D) section of liver treated with silibinin showing the normal histoarchitectural pattern of hepatic parenchyma (H and E, 40X).

Table 1: Changes in body weight, body weight gain, food intake, water intake and organ-body weight ratio in control and experimental rats.

Variables	Control				
	Initial (g)	Final (g)	Arsenic	Arsenic + Silibinin	Silibinin
Body weight	182±7.57	210±9.98	175±8.14	178±8.54	181±6.94
Body weight gain (%)	18.55	10.87	198±9.22	204±9.17	211±10.12
Food intake (g/100 g bw/day)	10.78±0.72 ^a	7.62±0.47 ^b	14.65±1.12 ^b	9.53±0.95 ^c	10.32±0.54 ^a
Water intake (mL/rat/day)	20.82±1.98 ^a	14.65±1.12 ^b	17.30±1.60 ^c	20.44±2.02 ^a	1.98±0.57 ^a
Organ-body weight ratio (%) liver	1.98±0.57 ^a	2.87±0.51 ^b	2.01±0.12 ^c	1.72±0.24 ^a	

DISCUSSION

In this present study, arsenic treatment induced hepatic injury via alterations of lipid profiles, after 28 days with noticeable alterations in its liver function. Lipids are precursors for hormones, and are used for energy storage, and they have a prominent role as messengers and regulators of inflammation (Watson, 2006). Moreover, lipids are one of the most susceptible targets of free radicals (Rajani and Purnima, 2009). This oxidative stress induced lipid peroxidation causes many pathological events. Increased level of TG also lead to many diseases like Diabetes mellitus, obesity, chronic renal disease, primary hyperlipoproteinemia carry high risk of cardiovascular disorder (CVD) (Ford, 2002). Hypertriglyceridemia in combination with abnormally low concentrations of HDL cholesterol (high density lipoprotein cholesterol) is one the most common atherogenic profile of lipid metabolism of high prevalence seen in Indian population (Enas and Mehta, 1995; Vijaya Padma *et al.*, 2012). Hyperlipidemia and

hypercholesterolemia are reported as the major risk factors in life style related diseases such atherosclerosis and related cardiovascular complications including cerebral paralysis and myocardial infarction (Chovaneikova and Simek, 2001). Prevention or treatment of such disorders can be achieved through diet and/or drug administration (Grundy *et al.*, 2004; LaRosa *et al.*, 1990). So the present investigation designed to prevent the As induced alterations in serum and hepatic lipid profiles.

Liver is the major target organ for As induced hepatotoxicity (Muthumani and Miltonprabu, 2012a), because metabolism of As involved only in liver. Increased levels hepatic marker enzymes (AST, ALT and ALP) indicate the permeability and damage and/or necrosis of hepatocytes (Goldberg and Watts, 1965). These membrane bound enzymes are unequally released into bloodstream depending on the pathological phenomenon (Sillanaukee, 1996). In the present study, we have found that As intoxication caused a significant increase in the activities of AST,

ALT and ALP which could be due to severe damage of membrane of hepatocytes and also these result correlated with our previous finding (Muthumani and Miltonprabu, 2012a). supplementation of SB along with As significantly normalized the levels of hepatic marker enzymes, which indicates the protective influence of SB on As induced alterations in membrane and that may reduce the hepatic dysfunction due to its membrane stabilization properties against ROS mediated oxidative hepatocellular injury (Basiglio *et al.*, 2009). Serum total cholesterol, LDL, VLDL and triglycerides significantly increases in the As treated rats followed by a significant decrease in the HDL cholesterol. A significant increase in the cholesterol and triglyceride values followed by a significant decrease in the phospholipid level was observed in the liver tissue of As treated rats which is an indication of severe As induced hyperlipidemia. As induced alterations in hepatic lipid profiles were reported earlier by Chattopadhyay *et al.* (2011). The results of the present investigation are in line with the above mentioned report. Moreover rats administered with SB along with As showed significantly reduced levels of the serum total cholesterol, LDL, VLDL, triglycerides and tissue total cholesterol and triglyceride whereas the serum HDL cholesterol and tissue phospholipid levels were significantly increased when compared with As treated rats. Mostly flavonoid compounds are free radicals scavengers and exhibits lipid peroxidation lowering effect by radical scavenging activity. SB is a phenolic flavonoid found in silymarin, isolated from the seeds or fruits of milk thistle of *Silibum marianum*. The structure of SB plays an important role in its antioxidant effect. SB encounters the As induced free radicals via carbonyl oxygen and OH groups (3-OH or 5-OH) to form the metal-oxygen bonds in the complexes (Naso *et al.*, 2011) and also confer higher stability to the radical form and to participate in electron donating to the free radicals. It was suggested that the antioxidant activity of SB exhibited by their structural features lead to neutralizing of As induced ROS. Lipophilic nature of SB also lead to distributed on the surface of the lipid bilayers as well as in the aqueous phase could scavenge free radicals. SB also showed antioxidant and free radical scavenging actions (Basiglio *et al.*, 2009). SB being a potent antioxidant exhibits scavenging effect on As induced free radicals and maintain serum and tissue lipid profile at near normal values.

Histopathological examination of liver revealed that As intoxication caused severe pathological alterations in the tissue. However, pre-treatment with SB could prevent the changes and also maintain the ultra structure almost similar to that of the normal control. More specifically SB exerted its protective action against As induced oxidative hepatic damage, probably through its capacity to quickly and efficiently scavenge lipid peroxy radicals before they attack membrane lipids and thereby maintain the normal hepatic architecture (Erlejan *et al.*, 2004).

CONCLUSION

From the above investigation it is obvious that As induced alteration in the lipid profile can be attenuated by SB. Our

study recommended the use of natural free radical scavenger SB in diet to reduce toxic effects of As.

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ABBREVIATIONS

As – Arsenic
 SB - Silibinin
 AST – Aspartate transaminase
 ALT – Alanine transaminase
 ALP – Alkaline phosphatase
 LDL – Low density lipoprotein
 VLDL – Very low density lipoprotein
 HDL – High density lipoprotein

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