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Beneficial role of silibinin in monitoring the cadmium induced hepatotoxicity in Albino Wistar rats

R. Srinivasan[1] and C. Ramprasath[2]

[1]Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Annamalainagar – 608 002, Tamil Nadu, India
[2]Biocontrol and Microbial metabolite lab, Centre for Advanced Studies in Botany, University of Madras, Maraimalai campus, Guindy, Chennai 600 025, Tamil Nadu, India

Abstract
Cadmium (Cd), an environmental toxic pollutant affects many organs in human beings specially the liver and kidney. In this study, Cd (3 mg/kg body weight (b.w.)) was subcutaneously administered to rats for 3 weeks, which shows significantly ($P<0.05$) increase in the activities of serum transaminases, alkaline phosphatase and lactate dehydrogenase, with significant elevation of lipid peroxidation along with significant ($P<0.05$) decreased in the levels of antioxidants in the liver. Oral administration of silibinin (SB) at 80 mg/kg b.w. significantly normalized the activities of serum hepatic enzymes and reduced the levels of lipid peroxidation and also restored the antioxidant defense in the liver when compared to other doses of SB (20 and 40 mg/kg b.w.). Histopathological analysis of liver was consistent with the biochemical findings. From this study we conclude the curative potential of SB against Cd- induced hepatic injury.

Keywords: Antioxidants, Cadmium, Lipid peroxidation, Liver, Silibinin

INTRODUCTION

Cadmium (Cd) a highly toxic metal which has been classified as human carcinogen by International Agency for Research on Cancer (Bertin and Averbeck, 2006). It is used in electroplating, paint, dyestuffs and mining industries and it is major threat to humans. In mammals, Cd targets the liver (Santos et al., 2004), brain (Shaikh and Tang, 1999), lungs (Horiguchi et al., 2000), gastrointestinal tract (Weisman, 1998) and kidney (El-Sharaky et al., 2007). Human beings are also exposed by various ways such as cigarette smoke, air pollution and food chain (Chen et al., 2007). Cd after entering into the body, it binds to albumin and erythrocytes in the blood and then it is transferred into tissues and organs, where it is bound to proteins of low molecular mass producing metallothioneins (George et al., 1996). The molecular mechanism that may be responsible for the toxicity of Cd involves oxidative stress disturbing the antioxidant defense system and by producing reactive oxygen species (ROS) (Thijssen et al., 2007) like hydrogen peroxide (H_2O_2), superoxide anion (O_2^{-}), hydroxyl radical (OH^{-}), which increase lipid peroxidation (LPO), change intercellular stability, damage deoxyribonucleic acid (DNA), membranes and cell death (Stohs et al., 2001). More beneficial effects were documented for combined treatment with chelating agent and antioxidants against Cd-induced oxidative stress in rats liver (Tandon et al., 2003).

Flavonoids are phenolic compounds widely distributed in plants, which were reported to exert multiple biological effects, including antioxidant and free radical scavenging abilities (Slaterand Eakins., 1975; Bors and Saran, 1987; Ne`gre-Salvayre and Salvayre, 1992). Flavonolignan silibinin (SB) (Fig. 1) is a major biologically active component of silymarin which are extracted from the seeds of milk thistle (*Silybum marianum* (L) *gaertn*). SB is present in numerous phytopreparations used in the prevention and treatment of various liver diseases and as protectants against a number of hepatotoxins (Flora et al., 1998) and mycotoxins. (Gallo et al., 2003). It possess multiple beneficial activities which are related to hepatoprotective (Ferenci et al ., 1989), metal chelating property (Pietrangelo, et al., 1995), effective antioxidant (Saller et al ., 2001), free radical-scavenging (Winterbourn, 2008), anticancer (Deep and Agarwal, 2007), chemoprotective (Comelli et al ., 2007), hypocholesterolemic (Skottova et al., 1999), neuroprotective (Kittur et al ., 2002) and estrogenic activity (Pliskova et al., 2008).

Eventhough the pharmacological properties of SB have been well established, the molecular mechanism of the antioxidative activity of SB and its derivative has not been systematically investigated and remains unclear. The active sites of SB is C-20 hydroxyl and have been identified their essential role in the interaction with radicals. (Gyorgy et al., 1992).

The present study was designed to demonstrate the hepatoprotective activity of the SB against the potent heavy metal (Cd) toxin induced hepatotoxicity in rats. For this, we aimed to measure the hepatic marker enzymes such as aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) and gamma gultamyl transferase (GGT). The prooxidant-antioxidant status of the liver was assessed by measuring the activities of the intracellular antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione -S- transferase (GST) and evaluating the levels of the ROS scavengers reduced glutathione (GSH) and the levels of the Vitamin-C, Vitamin-E and the end products of LPO (TBARS and LOOH).

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*Corresponding Author

R. Srinivasan
Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Annamalainagar – 608 002,
Tamilnadu, India

Tel: +91-4142 327735
Email: srinivasanrbiotch@gmail.com

MATERIALS AND METHODS

Chemicals

Silibinin, cadmium chloride and other fine chemicals were obtained from Sigma – Aldrich, Co. (St. Louis, Mo, USA). Commercial kits to estimate AST, ALT, ALP and LDH, were obtained from Agappe Diagnostics (I) Pvt. Ltd. (Kerala, India). All the other chemicals were of analytical grade obtained from a local firm (India).

Animals

Male albino Wistar rats of initial body weight 180-220 g were used in this experimental study. The rats were bred in the Central Animal House, Rajah Muthiah Medical College, Annamalai University (temperature 28 ± 2 °C; natural light-dark cycle). The rats had free access to drinking water and commercial standard pellet as diet (M/S. Pranav Agro Industries Ltd., Bangalore, India) and water ad *libitum*. The laboratory animal protocol used in this study was approved (Approval No: 610, 2009) by the Institutional Animal Ethical Committee (IAEC) at Annamalai University, Annamalainagar, India. In this experimental study, a total of 36 rats were used.

Experimental design

The total rats were randomly divided into six groups of six rats in each.

Group 1: Control rats (Vehicle treated)

Group 2: Normal rats orally administrated with SB (80 mg/kg b.w. /day) dissolved in 0.1% Dimethyl Sulphoxide (DMSO) for 3 weeks.

Group 3: Normal rats were subcutaneously received Cd as cadmium chloride (3 mg/kg b.w. / day) (Pari and Murugavel, 2005) in isotonic saline for 3 weeks.

Group 4: Rats subcutaneously received Cd (3mg/kg bw/day) followed by oral administration of SB (20 mg/kg b.w. /day) in 0.1% DMSO for 3 weeks.

Group 5: Rats subcutaneously received Cd (3mg/kg bw/day) followed by oral administration of SB (40 mg/kg b.w. /day) in 0.1% DMSO for 3 weeks.

Group 6: Rats subcutaneously received Cd (3mg/kg bw/day) followed by oral administration of SB (80 mg/kg b.w. /day) in 0.1% DMSO for 3 weeks.

At the end of the experimental period, animals in different groups were sacrificed by decapitation under pentobarbitone sodium (60 mg/kg body weight) anaesthesia. Blood samples were collected for separation of serum by the centrifugation at 2000xg for 20 minutes. The liver was dissected out, weighed and washed using chilled saline solution. Tissue was minced and homogenised (10%, w/v) in appropriate buffer (pH 7.4), and centrifuged (3000×g for 10 min). The clear supernatant was used for various biochemical assays.

Biochemical assays

Activities of serum marker enzymes

The activities of serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) were assayed by spectophotometrically according to the standard procedures using commercially available diagnostic kits (Agappe Diagnostics (I) Pvt. Ltd., Kerala, India). Gamma glutamyl transferase (GGT,) activity was determined by the method of Rosalki et al. (1970) using γ-glutamyl-p-nitroanilide as substrate.

Estimation of LPO

Lipid peroxidation (LPO) in liver was determined by measuring the levels of thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LOOH) by the method of Niehaus and Samuelsson (1968) and Jiang et al., (1992), respectively.

Determination of antioxidant activities.

The level of reduced glutathione (GSH) in the liver was estimated by spectrophotometric method based on the reaction with Ellman's reagent (19.8 mg dithionitrobenzoic (DTNB) in 100 ml of 0.1% sodium citrate) according to Moron et al., (1979). Ascorbic acid (vitamin C) and α- tocopherol (vitamin E) concentrations were measured by the method of Omaye et al., (1979) and Desai, (1984) respectively. The activities of superoxide dismutase (SOD) (Kakkar et al., 1984), catalase (CAT) (Sinha, 1972), glutathione peroxidase (GPx) (Rotruck et al., 1973) and glutathione-S-transferase (GST) (Habig et al., 1974) were also measured in liver tissue homogenate by spectrophotometrically.

Histopathological studies

The liver tissue samples fixed for 48 hours in 10% formalin were dehydrated by passing successfully in a different mixture of ethyl alcohol - water, cleaned in xylene and embedded in paraffin. Sections of liver (5-6 mm thick) were prepared and then stained with hematoxylin and eosin dye (H&E), and mounted in neutral DPX medium for microscopic observations.

Statistical analysis

All data's are expressed as mean ± SD of number of experiments (n=6). The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS version 11.5 (SPSS, Cary, NC, USA), and the individual comparison were obtained by Duncans' Multiple Range Test (DMRT). Values were considered statistically significant when $P<0.05$ (Duncan, 1957).

RESULTS

Biochemical analysis

Table 1 demonstrates the activities of serum hepatic enzymes namely AST, ALT, ALP, LDH and GGT and bilirubin in Cd and SB treated rats. The Cd-treated rats were showed significantly ($P<0.05$) increased in the activities of AST, ALT, ALP, LDH and GGT and bilirubin when compared to control rats. Oral administration of SB were significantly ($P<0.05$) normalized the levels of serum hepatic marker enzymes in a dose dependent manner. In this manner SB at 80mg/kg b.w. was more effective when compared with other two doses (20 and 40mg/kg b.w.). There was no significant changes were found in control or SB alone treated groups. Hence, 80mg/kg b.w. dose was followed for the further studies.

Table 1. Effect of SB on Cd induced serum biochemical changes in control and experimental rats

Groups	Control	Normal + SB (80 mg/kg)	Normal + Cd (3 mg/kg)	Cd (3 mg/kg) + SB (20 mg/kg)	Cd (3 mg/kg) + SB (40 mg/kg)	Cd (3 mg/kg) + SB (80 mg/kg)
AST (IU/L)	55.13 ± 4.25^{a}	54.86 ± 4.35^{a}	88.51 ± 7.83^{b}	79.52 ± 7.04^{c}	69.47 ± 5.14^{d}	62.41 ± 5.64^{e}
ALT (IU/ L)	24.98 ± 2.61^{a}	24.81 ± 2.05^{a}	52.46 ± 3.68^{b}	44.61 ± 2.68^{c}	36.48 ± 2.41^{d}	29.75 ± 2.05^{e}
ALP (IU/ L)	88.12 ± 5.86^{a}	87.66 ± 5.96^{a}	145.43 ± 8.83^{b}	130.09 ± 7.96^{c}	117.09 ± 8.09^{d}	98.31 ± 7.04^{e}
LDH (IU/ L)	107.09 ± 9.77^{a}	106.03 ± 9.05^{a}	169.52 ± 14.37^{b}	153.62 ± 12.46^{c}	139.46 ± 10.10^{d}	122.74 ± 11.26^{e}
GGT (IU/ L)	0.60 ± 0.01^{a}	0.59 ± 0.01^{a}	0.98 ± 0.09^{b}	0.88 ± 0.06^{c}	0.79 ± 0.04^{d}	0.68 ± 0.04^{e}
Bilirubin (mg/dl)	0.44 ± 0.04^{a}	0.48 ± 0.05^{a}	1.69 ± 0.09^{b}	1.27 ± 0.07^{c}	1.02 ± 0.08^{d}	0.68 ± 0.05^{e}

Values are mean ±SD for 6 rats in each group. $^{a-e}$ In each rows, means with different superscript letter differ significantly at p<0.05 (DMRT). Cadmium+ Silibinin (Cd + SB), aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) and gamma gultamyl transferase (GGT).

Table 2. Changes in the levels of lipid peroxidation markers in plasma and liver of control and experimental rats

Groups	Control	Normal + silibinin (80 mg/kg)	Normal + Cd (3 mg/kg)	Cd (3 mg/kg) + silibinin (80 mg/kg)
TBARS (mmoles/dl)				
Plasma	0.14 ± 0.01^{a}	0.12 ± 0.01^{a}	0.32 ± 0.03^{b}	0.17 ± 0.01^{c}
Liver	8.11 ± 0.08^{a}	8.06 ± 0.09^{a}	18.51 ± 1.69^{b}	10.02 ± 0.63^{c}
LOOH (mM/dl)				
Plasma	10.55 ± 0.67^{a}	9.76 ± 0.54^{a}	16.76 ± 1.42^{b}	11.55 ± 0.76^{c}
Liver	0.85 ± 0.06^{a}	0.84 ± 0.03^{a}	1.74 ± 0.23^{b}	1.07 ± 0.06^{c}

Values are mean ± SD for 6 rats in each group. $^{a-c}$ In each rows, means with different superscript letter differ significantly at p<0.05 (DMRT). Cadmium+ Silibinin (Cd + SB), thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (LOOH).

Table 3. Changes in the levels of non enzymic antioxidants in plasma and liver of control and experimental rats

Groups	Vitamin C		Vitamin E		GSH	
	Plasma	Liver	Plasma	Liver	Plasma	Liver
Control	2.01 ± 0.09^{a}	1.60 ± 0.07^{a}	1.20 ± 0.08^{a}	0.93 ± 0.06^{a}	20.94 ± 1.20^{a}	4.78 ± 0.25^{a}
Normal + SB (80 mg/kg)	2.08 ± 0.34^{a}	1.64 ± 0.07^{a}	0.98 ± 0.07^{b}	0.75 ± 0.05^{b}	22.05 ± 1.56^{a}	4.80 ± 0.43^{a}
Normal+ Cd (3 mg/kg)	0.98 ± 0.07^{b}	0.75 ± 0.05^{b}	$0.76 \pm 0.06+$	0.38 ± 0.01^{b}	12.85 ± 1.09^{b}	2.25 ± 0.16^{b}
Cd (3 mg/kg) + SB (80 mg/kg)	1.75 ± 0.08^{c}	1.36 ± 0.06^{c}	$12.85 \pm 1.09+$	2.25 ± 0.16^{b}	18.63 ± 1.01^{c}	5.98 ± 0.22^{c}

Protective effect of SB on Cd induced experimental peroxidative damage is shown in Table. 2. Hepatic and plasma LPO was enhanced after Cd intoxicated rats (P<0.05). Oral administration of SB therapy significantly inhibited LPO (P<0.05) and reduced the peroxidative stress in liver.

Effect of SB and Cd on enzymic antioxidants is shown in Fig. 2, 3, 4, 5. Cd intoxication diminished the status of enzymic antioxidants by decreasing the activities of hepatic SOD, CAT, GPx and GST (P<0.05). Oral administration of SB restored the activities of SOD, CAT, GPx and GST (P<0.05) towards control.

Table 3 presents the non-enzymic antioxidant status in terms of GSH, vitamin C and vitamin E. Cd intoxication significantly inhibited the activities of GSH, vitamin C and vitamin E (P<0.05). SB therapy enhanced the production of GSH, vitamin C and vitamin E towards control (P<0.05).

Histopathological studies

The histopathological studies of the liver in Cd-intoxicated rats showed microvesicular fatty change of hepatocytes and periportal inflammatory cell infiltration (Figure 2C), whereas the oral administration of SB to Cd-injected rats showed the reduction of fatty changes with mild portal inflammation in hepatocytes (Figure 2D). The control (Figure 2A) and SB alone (Figure 2B) treated rats showed normal architecture of the liver.

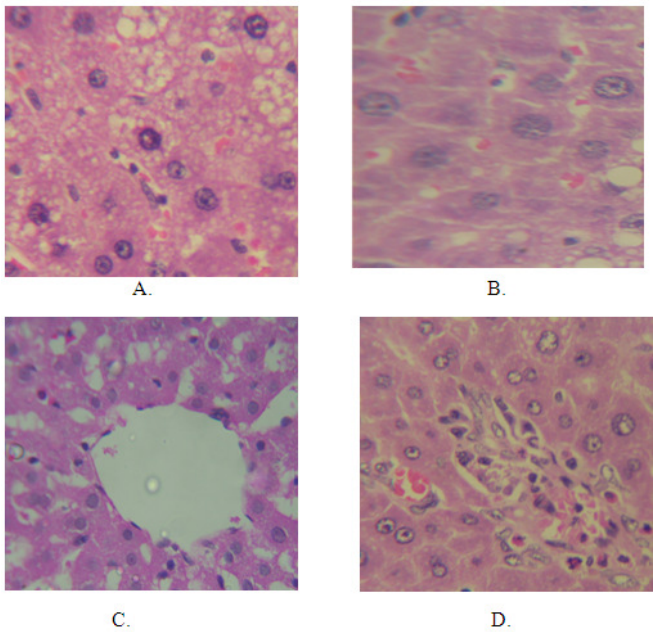

Fig 2. (A) Shows histology of control rat liver architecture (400x), (B) Demonstrate the histology of SB (80 mg/kg) alone treated rat liver cells which appear near to normal (400x), (C) illustrate the Cd (3 mg/kg) alone treated rat histopathology which shows microvesicular fatty change of hepatocytes and periportal inflammatory cell infiltration (400x), (D) shows the Cd (3 mg/kg) + SB (80 mg/kg) treated rat which exhibit the reduction of fatty changes with mild portal inflammation in hepatocytes (400x).

DISCUSSION

Cd- induced liver damage is well-documented, which involves changes in many biochemical and physiological process. The well-established efficiency of flavonolignan in several *invitro* and *invivo* models showed the oxidative stress-mediated hepatocellular injury in experimental rats (Comelli et al., 2007). So we aimed to study its efficacy on Cd- induced hepatotoxicity in rats.

To assess the Cd toxicity in the chosen tissues through studying the changes of the biochemical profiles of certain vital hepatic enzymes activities (Sujatha et al., 1999). AST and ALT which are the hepatic marker enzymes mainly elevated during hepatic injury. The functional state of the liver is indicated by the changes in the levels of AST and ALT in serum. ALP is a membrane bound enzyme and its alteration produce derangement in the transport of metabolites (Ahmed et al., 1999). LDH is an intracellular enzyme, is also an indicator for cell damage by elevation of LDH in serum (Kim et al., 2001). After Cd treatment the cell membrane is damaged, resulting in an increased release of functional enzymes in serum, which gives the indication of hepatic injury caused by Cd. Serum GGT, has been widely used as an index to assess liver dysfunction. Recent studies indicating that serum GGT might be useful in studying oxidative stress-related issues. The products of the GGT reaction may themselves lead to increased free radical production, particularly in the presence of iron (Lee et al., 2004). In Cd treated rats, the significant elevation of serum GGT might be related to increased oxidative stress induced by Cd. Oral administration of SB (80 mg/kg b.w.) significantly normalized the levels of serum hepatic enzymes in Cd-intoxicated rats, which indicate the interference of SB with Cd-induced alterations in cell membrane, that reduce the hepatic dysfunction and leakage of hepatic marker enzymes in blood.

Cd–induced oxidative damage has been established by increased LPO and decrease of antioxidant levels, which indicates the preventing from the oxidative damaged products (Kelley et al., 1999). The observed increase in the level of TBARS and hydroperoxides in Cd toxicity is generally thought to be the consequence of increased production and liberation of tissue lipid peroxides, due to increased formation of free radicals as a result of Cd accumulation in the liver (Waisberg et al., 2003). Administration of SB to Cd-treated rats, significantly decrease lipid peroxides due to the ability of SB to scavenge the free radicals, suggesting the bioactivity of SB to directly react with various reactive oxygen species (ROS). The free radical scavenging property of SB has been already well established in experimental rats (Winterbourn, 2008).

As a result of Cd-toxicity, a remarkable diminution of tissue and circulating enzymic antioxidants such as SOD, CAT, GPx and GST are noticed (Casalino et al., 2002). After oral administration of SB (80mg/kg b.w.) to Cd-treated rats the level of enzymic antioxidant is significantly increased in the liver. SB inhibits the membrane LPO, which induce the expression of SOD in astrocytes (Dehmlow, 1996). The mechanism action of SB enhancing the level of GST simultaneously it increase the level of GSH (Zhao and Agarwal, 1999).

In Cd exposed rats, a depletion of non-enzymic antioxidants in liver tissue, which includes GSH, vitamin C and vitamin E, have been observed (Pari and Murugavel, 2005). The main route of Cd intoxication is exhaustion of glutathione and binding to the SH group of proteins. The first defense mechanism against Cd toxicity is provided by GSH, which efficiently binds with Cd via its free sulphydryl groups, and eventually become oxidized to oxidized glutathione (Valko, 2005). Vitamin C is a major preventive antioxidant in the cells and body fluids, which scavenges the free radicals and serves as a metabolic marker of Cd toxicity (Pharikal et al., 1988).

Vitamin E is a lipophilic antioxidant, which plays a crucial role

in detoxifying the free radicals developed through Cd induced oxidative damage in the liver. The diminution of sulphydryl assets seems to be an important mechanism for oxidative stress caused through indirect mechanism of Cd (Stoh and Bagchi, 1993). As a result of decrease in the levels of vitamin C and vitamin E which leads to increased susceptibility of the tissues to free radical damage through Cd intoxication (Sunitha, 2001). SB directly scavenges the free radicals through chelating Cd and moderates the expenditure of non-enzymic antioxidants endogenously. The thiol compounds possess antioxidant actions, which include reducing power and metal ion chelating effect, in addition it improved the alteration in the intracellular redox potential which in turn can regulate the activity of several transcription factors and it results in modulation of cellular functions (Sun and Oberley, 1996). In addition, the active sites of SB C-20 hydroxyl groups may contribute to improve the tissue thiol pools, which could be associated with a reduction of Cd-induced oxidative threat, and increased antioxidant status in SB administrated Cd-treated rats.

Chelation of Cd reduces the oxidative stress, reverses the antioxidant level and glutathione metabolizing enzymes activities due to the presence of hydroxyl groups in SB and it react with free radicals.

Along with the results of biochemical tests, the histopathological observations imply that Cd accumulation leads to serious changes in the histology of the liver, including microvesicular fatty change, inflammatory cell infiltration, focal necrosis and sinusoidal dilation, thus posing a health risk, which has been also reported previously (Tzirogiannis et al., 2004). Administration of SB reduced the histological alterations in the liver caused by Cd intoxication, which may be due to the chelation of Cd content in the treatment. In addition, SB administrated to Cd-intoxicated rats contributed to improve the antioxidant defense and depletion of Cd, it could be the reason for protection of liver architecture and functions, which compared with hepatic markers.

In conclusion, our study proposes that SB may play a beneficial role to reduce the toxic effects of Cd-induced damage in liver, which could be due to its antioxidant nature scavenge the free radicals and by metal chelating activities. Future studies are required to elucidate the specific mechanisms by which SB protect the Cd-induced toxicity in experimental rats.

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