Research Paper

Hepatoprotective Effects and Safety Evaluation of Coumarinolignoids Isolated from *Cleome viscosa* Seeds

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The aim of the present work was to investigate the *in vivo* hepatoprotective potential of coumarinolignoids (cleomiscosins A, B, and C) isolated from the seeds of *C. viscosa*. The study was performed against CCl_4-induced hepatotoxicity in albino rats. Rats were divided into four groups. The animals of group I served as normal and was given only vehicle. Group II served as toxin control and administered with CCl_4 (50% solution liquid paraffin, 2 ml/kg intraperitoneally). The animals of group III received coumarinolignoids (50 mg/kg) for six days orally as well as CCl_4 (2 ml/kg) on 4^{th} day i.p. Similarly animals of group IV received silymarin (50 mg/kg) for six days orally as well as CCl_4 on 4^{th} day i.p. On 7^{th} day various parameters viz. serum glutamyl oxaloacetic transaminase, serum glutamyl pyruvate transaminase, serum alkaline phosphatase, serum bilirubin, liver glycogen were estimated and histopathology was performed. Additionally, acute oral toxicity of the said coumarinolignoids was carried out in swiss albino mice. The coumarinolignoids were found to be effective as hepatoprotective against CCl_4-induced hepatotoxicity as evidenced by *in vivo* and histopathological studies in small animals. Safety evaluation studies also exhibit that coumarinolignoids are well tolerated by small animals in acute oral toxicity study except minor changes in red blood cell count and hepatic protein content at 5000 mg/kg body weight as a single oral dose. Coumarinolignoids which is the mixture of three compounds (cleomiscosin A, B and C) is showing the significant protective effects against CCl_4-induced hepatotoxicity in small animals and also coumarinolignoids are well tolerated by small animals in acute oral study.

Key words: Coumarinolignoids, *Cleome viscosa*, CCl_4, hepatoprotection, safety evaluation

Cleome viscosa Linn. (Capparidaceae) is an annual herb with yellow flowers and strong penetrating odor, which occurs as a weed in rain fed soils. The genus *Cleome* Linn. consists of about 140 species of herbs and under shrubs, distributed in tropical and sub-tropical zones. The seeds are small, dark brown or black and granular. They are reported to have rubefacient, vesicant and anthelmintic properties. They resemble mustard seeds in action and a poultice made from them is efficacious as a counter-irritant in chronic painful joints. They are effective in round worm infections. The seeds are occasionally used as a condiment in curries[1]. In the Ayurvedic system of medicine, this plant is used in the treatment of fever, inflammation, liver diseases, bronchitis, diarrhea and infantile convulsions[2]. The seeds of this plant are widely said to be anthelmintic[3]. The rural people use the fresh juice of the crushed seed for the treatment of infantile convulsions and in mental disorders[4], Antipyretic[5], analgesic[6] and psychopharmacological actions[7] of the whole plant extracts have been reported. Recently, *in vivo* immunomodulatory activity of the coumarinolignoids from this plant also has been reported[8]. The seeds of *Cleome viscosa* have been investigated and they were found to be the source of an interesting class of compounds known as coumarinolignoids. Four such compounds, cleomiscosin A, B, C and D have been isolated from the seeds of the plant and cleomiscosin A, B and C possess *in vitro* antihepatotoxic activities[9,10]. There have been no reports on the *in vivo* hepatoprotective potential of these cleomiscosins either individually or in combination. Therefore, the aim of the present work was to investigate the *in vivo* hepatoprotective potential of these coumarinolignoids in combination because it was difficult to get the cleomiscosin A, B and C separately in sufficient quantity to carry out the *in vivo* studies.

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MATERIALS AND METHODS

Isolation of coumarinolignoids:

The seeds of *C. viscosa* Linn. were collected from the National Gene Bank for Medicinal and Aromatic Plants, CIMAP, Lucknow, India and the voucher specimens are available for authentication. Seeds of *C. viscosa* (10 Kg) were used as starting material and the mixture of coumarinolignoids (cleomiscosin A, B and C) was obtained by following the methodology as described by Bawankule *et al.*[8]. Briefly, air dried pulverized seeds of *Cleome viscosa* (10 kg) were defatted with light petroleum (10 l×3) for 72 h. The defatted material was then exhaustively extracted with methanol (10 l×3) and concentrated to a viscous extract (900.00 g). It was adsorbed onto celite (1000 g), dried at room temperature for 24 h and then packed in a cheese cloth and extracted with toluene (10 l), followed by ethyl acetate (10 l) and methanol (10 l). The toluene and ethyl acetate fractions (101.00 g and 406.00 g, respectively) were mixed together, concentrated, and chromatographed over silica gel (60-120 mesh) using light petroleum. The column was eluted with mixtures of light petroleum–ethyl acetate in the ratio of 1:1 and 1:3, successively. From the above two eluants, on concentration, amorphous solids precipitated, which were removed by filtration and washed with light petroleum:ethyl acetate (1:1) to give a mixture (5.01 g) of cleomiscosins A, B and C in the ratio 21:25:4, respectively. The representative HPLC chromatogram of mixture of coumarinolignoids has been shown in fig. 1.

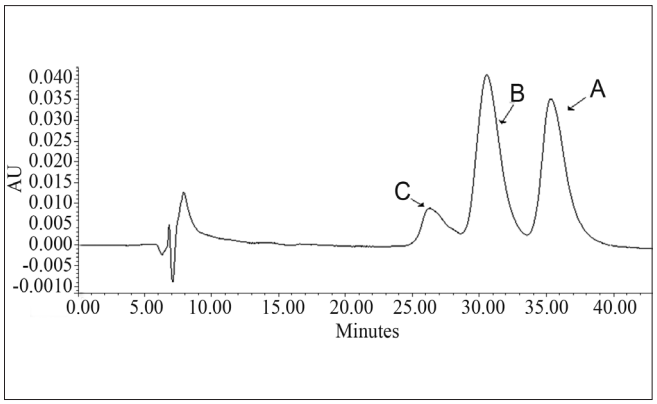

Fig. 1: HPLC chromatogram of the mixtures of coumarinolignoids. A-cleomiscosin A, B- cleomiscosin B and C- cleomiscosin C [Waters RP-18 column (250 × 4.6 mm i.d. with 5.0 μm particle size) solvent system: (ACN:MeOH) (1:2) (v/v) (0.5 % CH_3COOH in H_2O) in the ratio of 40:60 at a flow rate of 0.4 ml/min.] Photodiode array (PDA) detector was used and peaks have been detected at 326nm.

Animals:

Male Charles Foster rats (150±10 g) were the experimental subjects maintained under controlled conditions (temperature 25±2º; relative humidity 50%±5%; 12 h light/dark cycle). The animals were maintained on certified pelleted rodent diet (Dayal Industries, Lucknow, India). Water was provided *ad libitum*. Institutional Animal Ethics Committee approved the animal experiments and the guidelines for Animal care were followed as recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

In vivo hepatoprotective activity studies:

The hepatoprotective studies of coumarinolignoids were performed against carbontetrachloride induced hepatotoxicity in albino rats by using the model described by Yadav and Dixit[11] with some modifications. The rats were divided into four groups consisting of six animals each. The animals of group I served as normal and was given only vehicle (0.7% CMC suspension 1 ml/kg) for 6 days. The animals of group II served as toxin control and were administered with CCl_4 (50% solution of CCl_4 in liquid paraffin, 2 ml/kg, intraperitoneally) on 4th day and vehicle on rest of the days. The animals of group III received coumarinolignoids (50 mg/kg) for six days orally as well as CCl_4 (2 ml/kg) on 4th day i.p. Similarly animals of group IV received silymarin (50 mg/kg) for six days orally as well as CCl_4 on 4th day i.p. The detailed dosage regimen is given in Table 1.

Assessment of hepatoprotective activity:

On 7th day, blood was collected from the retro

TABLE 1: DOSAGE REGIMEN OF COUMARINOLIGNOIDS AND TOXIN TO EXPERIMENTAL ANIMAL GROUPS

Groups	Day(s)					
	1	2	3	4	5	6
A	Veh	Veh	Veh	Veh	Veh	Veh
B	Veh	Veh	Veh	Veh+CCl_4	Veh	Veh
C	Coumarino	Coumarino	Coumarino	Coumarino+CCl_4	Coumarino	Coumarino
D	Sily	Sily	Sily	Sily+CCl_4	Sily	Sily

Veh= Vehicle, Coumarino.= Coumarinolignoids, Sily= Silymarin. On day 7 Blood was collected, animals were sacrificed and liver was isolated

orbital plexus using hemocrit capillaries (Himedia). The blood was kept at 4ºC for 30 minutes followed by centrifugation at 3000 rpm at 8º for 7 min. The supernatant containing the serum was collected and analyzed for various biochemical parameters, i.e. serum glutamyl oxalacetic acid transaminase (SGOT), serum glutamyl pyruvate transaminase (SGPT)[12], alkaline phosphatase (ALKP)[13], serum bilirubin (SBLN)[14] by using enzymatic kits supplied by Bayer Diagnostics, India.

Liver glycogen and histopathological studies:

After collecting the blood from each animal, they were sacrificed by cervical dislocation and liver was separated, washed in Ringer's solution and soaked in filter paper. Immediately the liver was stored at -20º temperature and used for estimation of liver glycogen[15] and histopathological studies. The hepatoprotective activity was confirmed through histopathological studies on liver of rats. Liver tissues were cut into 5 µm thick slices using Leica Cryotome and fixed with 10% neutral formalin. Tissue sections of 5 µm were stained with aqueous hematoxylin and alcoholic eosin (H and E), and were observed with a light microscope Leica DMLB 2 for histopathological changes.

Acute toxicity study of coumarinolignoids in Swiss albino mice:

In view of potent hepatoprotective activity of coumarinolignoids in *in vivo* model, acute oral toxicity of the same was carried out in Swiss albino mice for its further development into drug product. Experiment was conducted in accordance with the Organization for Economic Co-operation and Development (OECD) test guideline No 423 (2001). For the study, 30 mice (15 male and 15 female) were taken and divided into five groups comprising 3 male and 3 female in each group, weighing between 20-25 g each. The animals were maintained at 22±5º with humidity control (50±5%) and also on an automatic dark and light cycle of 12 h. The animals were fed with the standard rat feed and provided *ad libitum* drinking water. Mice of group 1 were kept as control and animals of groups 2, 3, 4 and 5 were kept as experimental. The animals were acclimatized in the experimental environment prior to the actual experimentation. The test compound was suspended in 0.7% carboxymethyl cellulose and was given at 2000, 3000, 4000 and 5000 mg/kg body weight orally to animals of groups 2, 3, 4 and 5 respectively on Day 1. Control animals received only vehicle. All the animals were sacrificed on 7th day after the experimentation.

The animals were checked for mortality and any signs of ill health at hourly interval on the day of administration of drug and there after a daily general case side clinical examination was carried out including changes in skin, mucous membrane, eyes, occurrence of secretion and excretion and also responses like lachrymation, pilo-erection respiratory patterns etc. Also changes in gait, posture and response to handling were also recorded[16]. In addition to observational study, body weights were recorded, blood and serum samples were collected from all the animals on 7th day after experiment and were analyzed for total RBC, WBC, differential leucoyte count, haemoglobin percentage and biochemical parameters like total cholesterol, triglycerides, creatinine, SGPT and SGOT activity. The animals were then sacrificed and were necropsied for any gross pathological changes. Weights of vital organs like liver, heart, kidney etc. were recorded[17].

Statistical analysis:

Results are presented as Mean±S.E.M. Statistical analysis was performed using student's '*t*' test and one-way analysis of variance (ANOVA), wherever appropriate with the help of Graph Pad Prism 4.0 software. Difference between groups were considered to be statistically significant at $P<0.05$[18,19].

RESULTS

Hepatoprotective activity:

The results obtained for hepatoprotective activity

TABLE 2: BIOCHEMICAL PARAMETERS FOR THE HEPATOPROTECTIVE STUDIES OF COUMARINOLIGNOIDS

Parameters	Control	CCl_4	Coumarinolignoids+CCl_4	Sily+CCl_4
SGOT (U/l)	52.4±3.62	114.8^a±6.10	85.4^b±5.62	84.8^b±6.90
SGPT (U/l)	38.5±2.20	58.4^a±3.54	42.8^b±2.72	42.2^b±2.44
ALKP (U/l)	91.7±6.48	185^a±19.34	120.2^b±2.41	113.4^b±12.01
Total bilirubin (mg/dl)	0.25±0.06	0.66^a±0.12	0.19^b±0.03	0.28^b±0.04
Liver glycogen (mg/g of liver)	9.12±1	2.96^a±0.52	8.08^b±1.23	8.81^b±1

n=6, the values are mean±SEM. 'a' exhibit significant ($P<0.05$) changes from control. 'b' exhibit significant ($P<0.05$) changes when compared to toxin

in the experiment are presented in Table 2. Administration of CCl_4 to rats caused significant increase in the levels of serum enzymes like SGOT, SGPT, ALKP, and SBLN compared to treated rats. It also caused the significant change in biochemical parameters estimated from liver, viz., liver glycogen.

Results presented in Table 2 indicate that the levels of serum enzymes namely SGOT, SGPT, ALKP and SBLN were affected after treatments of rats with coumarinolignoids and silymarin when compared with toxin groups. SGOT levels were significantly ($P \leq 0.05$) reduced in treated groups, which were having CCl_4 induced hepatotoxicity. Similar results were obtained for SGPT and ALKP enzyme levels estimated from serum. Total bilirubin levels were also estimated from serum in all groups of animals. Coumarinolignoids and silymarin both significantly ($P \leq 0.05$) reduced the serum bilirubin levels in CCl_4 induced hepatotoxicity groups. Liver glycogen levels were significantly ($P \leq 0.05$) increased in animals administered with coumarinolignoids and silymarin and having CCl_4 induced hepatotoxicity. Grossly, the recovery was found to be comparable to silymarin (Table 3).

A comparison of liver section of normal animals (fig. 2a) with CCl_4 treated animals shows that the liver cells of rats intoxicated with CCl_4 have high degree of damage, as characterized by the ruptured central vein, cell vacuolation, pyknotic and degenerated nuclei and cords of hepatic cells that are disrupted (fig. 2b). The normal architecture of liver is completely damaged. The sinusides were observed to be badly damaged and their wall was broken at places with wide spaces formed at some sinusoids. The hepatic cells of rats treated with coumarinolignoids and silymarin and intoxicated with CCl_4 were radially arranged. The vacuolation was present, but was very much similar to that of normal (figs. 2c and 2d). The intralobular vein was almost normal in structure but damaged at one or two places in the wall. The hepatic cells were mostly normal but with few vacuoles and some damaged cells, but no pyknosis in the nucleus could be seen.

TABLE 3: PERCENT RECOVERY OF SERUM PARAMETERS

Parameters	Coumarinolignoids	Silymarin
SGOT	47.11±3.17	48.04±2.27
SGPT	78.39±4.23	81.40±4.16
ALKP	69.45±3.34	76.74±3.29
Total Bilirubin	114.63±4.72	92.68±4.58

For each group *n*=6, the values are Means±SEM, Formula for calculation of liver protection expressed as percent recovery is; (toxin group-treated group/toxin group-control group) × 100

Acute toxicity study:

No morbidity and mortality was found during the entire experimental observation. However, group of animals receiving doses at 3000 mg/kg body weight and above showed transient sedation, depression and inactivity for a period of 30 min which then subsided in next 60 min. Non-significant changes were observed in most of the parameters studied like gain in body weight, haematological parameters, and biochemical parameters when compared the respective control values (Table 4). However, significant decrease in total RBC count was observed in experimental groups treated with coumarinolignoids at 3000, 4000 and 5000 mg/kg when compared to respective control. Similarly, significant decrease in hepatic protein content was observed in experimental group receiving coumarinolignoids at 5000 mg/kg body weight, when compared to control. Gross pathological and vital organ weight measurement also did not show any significant changes (figs. 3, 4 and 5).

DISCUSSION

The ability of a hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms, which have been disturbed by a hepatotoxin, is the index of its protective effect. Protection of hepatic damage caused by carbontetrachloride administration has been widely used as an indicator of liver protective activity of drugs in general[20].

Serum glutamyl oxalacetic acid transferase (SGOT), Serum glutamyl pyruvate transferase (SGPT), Alkaline phosphatase (ALKP) and Total serum bilirubin (SBLN) in serum have been reported to be sensitive indicator of liver injury[21]. The disturbance in the transport function of the hepatocytes as a result of hepatic injury, causes the leakage of enzymes from cells due to altered permeability of membrane[22]. This results in decreased levels of GOT, GPT and alkaline phosphatase in the hepatic cells and a raised level in serum. The present study revealed a significant increase in the levels of SGOT, SGPT, SALKP and SBLN after exposure to CCl_4, indicating considerable hepatocellular injury. Administration of coumarinolignoids and silymarin attenuated the

TABLE 4: SAFETY EVALUATION OF COUMARINOLIGNOIDS ON MICE

	Coumarinolignoids (mg/kg, onetime oral)				
Parameters	**control**	**2000**	**3000**	**4000**	**5000**
Body weight (g)	27.63±1.84	28.99±1.87	28.20±2.41	29.23±1.91	29.37±1.25
Hemoglobin (g/dl)	10.28±0.83	11.70±0.59	11.87±1.13	12.6±0.69	11.88±0.51
RBC (millions/ml)	4.80±0.35	4.24±0.64	2.77±0.35*	2.59±0.22*	2.85±0.19*
WBC(1000/ml)	13.20±1.41	13.26±1.68	10.87±0.98	14.15±1.47	17.33±2.41
SGPT (U/L)	9.81±0.95	9.43±2.56	7.43±1.45	12.84±5.70	16.59± 2.88
SGOT(U/L)	28.94±2.95	34.05±2.50	26.67±1.96	30.50±7.52	20.82±2.14
ALKP(U/L)	201.3±21.51	232.3±49.43	323.6±54.88	332.3±40.36	350.5±48.91
Creatinine (mg/dl)	0.86±0.15	0.48±0.07	0.5±0.09	0.79±0.09	0.48±0.05
LPO (µ mol/mg protein)	14.84±2.93	13.37±4.02	8.68±1.30	13.74±4.72	9.15±1.47
Triglycerides (mg/dl)	81.22±11.11	64.29±8.30	58.46±4.50	59.67±5.36	53.51±2.95
Serum Protein (mg/ml)	581.3±30.76	523.6±16.42	511.6±58.64	516.8±18.70	484.1±29.25
Liver Homogenate Protein (mg/ml)	2581±286.8	2209±238.8	2277±212.5	1977±295.3	1457±120.9*
Serum Cholesterol (mg/dl)	92.49±7.68	91.74±9.38	67.64±15.70	88.00±3.45	91.98±4.40
GSH (p mol/mg protein)	27.96±4.17	25.57±6.25	27.33±5.58	22.48±6.41	15.58±1.30
Albumin (g/dl)	2.99±0.10	3.00±0.08	2.58±0.05	3.23±0.08	3.12±0.04
Bilirubin (mg/dl)	0.25±0.06	0.13±0.03	0.35±0.10	0.47±0.02	0.21±0.01

Values are Mean ±SEM, * $P<0.05$, compared to respective control. $n=6$

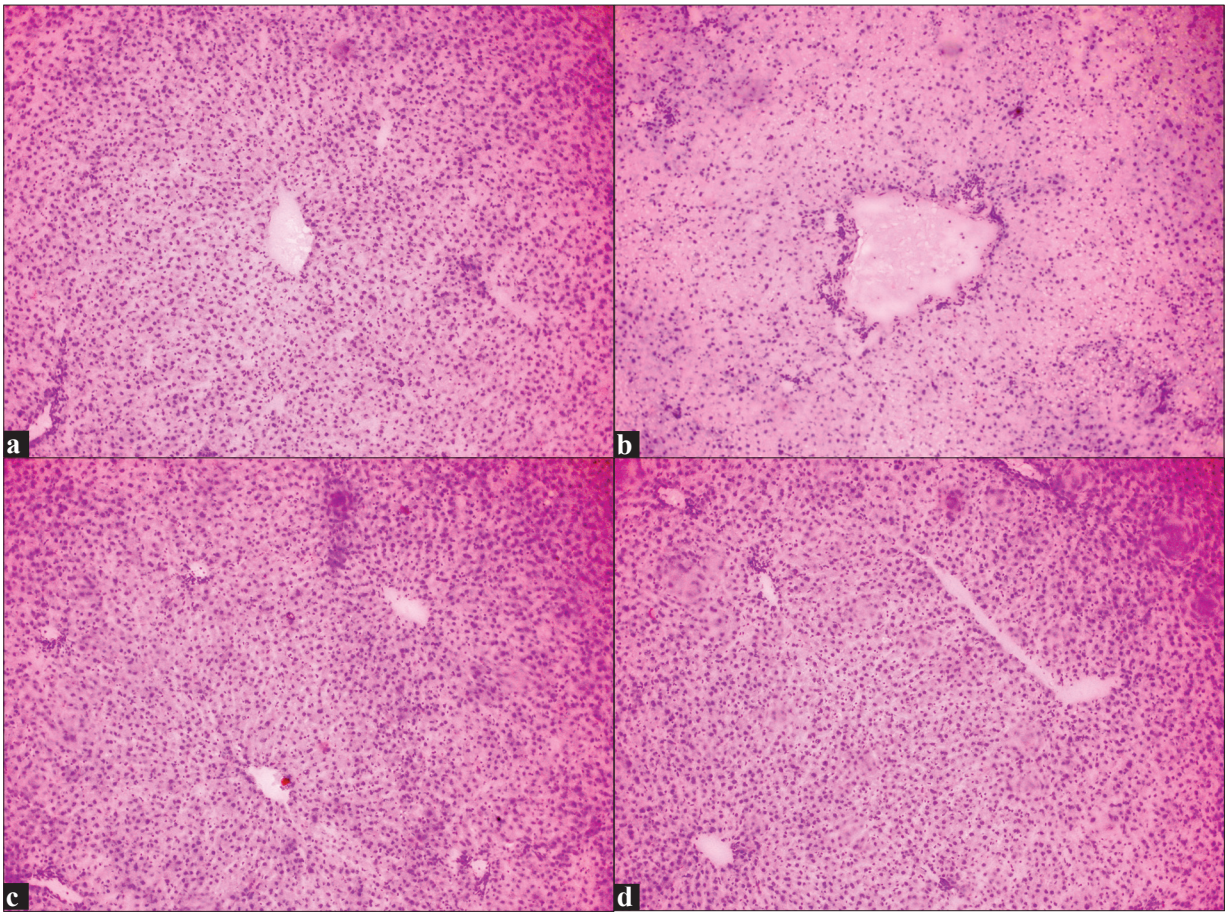

Fig. 2: Histopathological studies of coumarinolignoids.
(a) Liver cells of normal rat (b) Liver cells of rat intoxicated with CCl_4 (c) Liver cells of rat treated with coumarinolignoids+CCl_4 (d) Liver cells of rat treated with silymarin+CCl_4

increased levels of the serum enzymes, produced by CCl_4 and caused a subsequent recovery towards normalization. These recoveries have been presented in Table 3 in the form of percent recovery[11] of

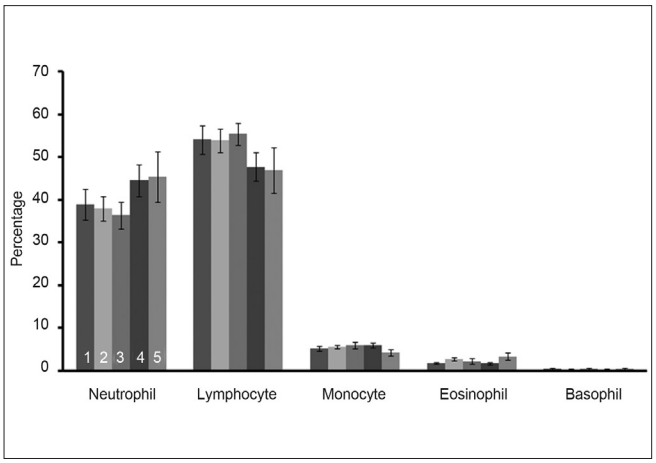

Fig. 3: Effect of coumarinolignoids on differential leucocyte count in mice.
1- Control, 2- 2000 mg/kg, 3- 3000 mg/kg, 4- 4000 mg/kg, 5- 5000 mg/kg

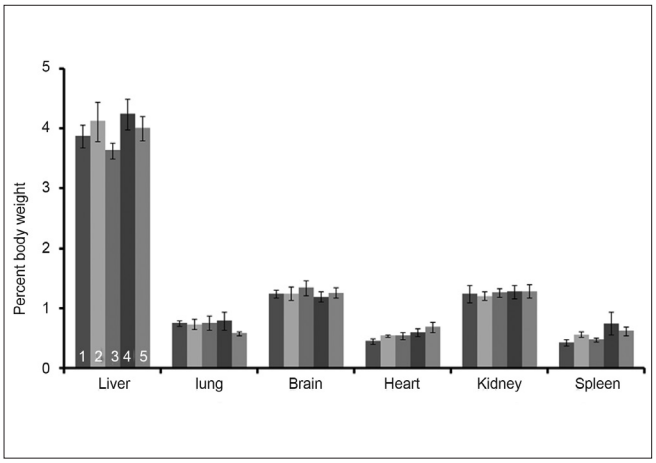

Fig. 5: Effect of coumarinolignoids on relative organ weight in mice.
1- Control, 2- 2000 mg/kg, 3- 3000 mg/kg, 4- 4000 mg/kg, 5- 5000 mg/kg

serum parameters by different test samples, where it was found that coumarinolignoids and silymarin, both are showing significant recovery of serum parameters. Coumarinolignoids are showing higher recovery of total bilirubin in comparison to silymarin (114.63±4.72 and 92.68±4.58, respectively). This suggested that coumarinolignoids isolated from *Cleome viscosa* and silymarin are able to condition the hepatocytes, so as to cause accelerated regeneration of parenchyma cells, thus protecting against membrane fragility and decrease the leakage of marker enzymes into the circulation. Silymarin is a known hepatoprotective compound. It is reported to have a protective effect on the plasma membrane of hepatocytes[23].

The primary function of liver is to store the energy in the form of glycogen. The liver glycogen content is directly proportional to the physiological condition

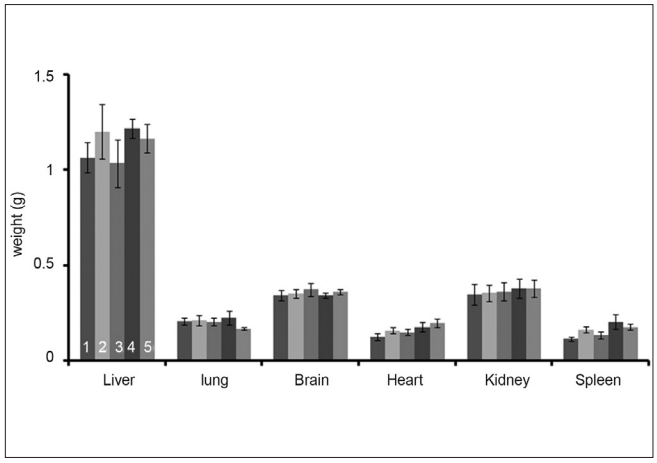

Fig. 4: Effect of coumarinolignoids on absolute organ weight in mice.
1- Control, 2- 2000 mg/kg, 3- 3000 mg/kg, 4- 4000 mg/kg, 5- 5000 mg/kg

of the liver. In our studies, the coumarinolignoids and silymarin, both are maintaining the glycogen level significantly higher than the toxin (CCl_4) group.

The histopathological studies are direct evidence of efficacy of drug as protectant. The section of the liver treated with coumarinolignoids and CCl_4 reveals significant hepatoprotective activity. Almost negligible damage to a few hepatocytes present in the close vicinity of intralobular vein is observed. Endothelium lining is almost smooth except at one or two places. Hepatocytes show normal appearance, only some cells show higher numbers of vacuoles in the cytoplasm but no pyknosis in the nucleus could be seen. The results of histopathological parameters also support the results of biochemical parameters and explain the hepatoprotective effect of coumarinolignoids in CCl_4 induced hepatotoxicity. Interestingly, coumarinolignoids on acute oral toxicity showed no observable change except decrease in RBC count and hepatic protein content beyond dose level of 5000 mg/kg body weight. In conclusion, it can be said that coumarinolignoids which is the mixture of three compounds (cleomiscosin A, B and C) is showing significant protective effects against CCl_4 induced hepatotoxicity in small animals. Besides, coumarinolignoids are well tolerated by small animals in acute oral toxicity studies.

ACKNOWLEDGEMENTS

The authors are thankful to Department of Biotechnology (DBT), India for providing financial assistance to the project and Central Institute of Medicinal and Aromatic Plants (Council of Scientific and Industrial Research) for providing infrastructure facilities.

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Accepted 24 December 2010
Revised 15 December 2010
Received 28 April 2010
Indian J. Pharm. Sci., 2010, 72 (6): 759-765