

HEPATOPROTECTIVE ACTIVITY OF THE OF ROOTS OF *LIMNANTHEMUM CRISTATUM* AGAINST CARBON TETRACHLORIDE-INDUCED INJURY IN RATS

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Summary

*Limnanthemum cristatum* (Roxb.) Griseb is described as a hepatoprotective in Indian traditional medicine. However, there are no reports available in the modern medicine regarding its usefulness as a hepatoprotective agent. In the present study the hydroalcoholic extract of the roots of *Limnanthemum cristatum* (Roxb.) Griseb (LC), at doses 250 and 500 mg/kg were screened for its hepatoprotective activity against CCl<sub>4</sub> (1 ml/kg, i.p) induced hepatotoxicity in male Wistar rats, when administered orally. The hepatoprotective activity was assessed using various biochemical parameters such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, serum total bilirubin, direct bilirubin, total protein. Tissue antioxidant parameters viz.lipid peroxidation, catalase and reduced glutathione were estimated and a histopathological study of liver tissue was carried out. Silymarin (25 mg/kg), a known hepatoprotective drug, was used as a standard for comparison. The results of acute toxicity tests revealed that the extract was safe. The *in-vivo* study revealed that the substantially elevated enzymatic levels of serum transaminases, alkaline phosphatase, direct bilirubin, indirect bilirubin and triglycerides were significantly improved towards normalization by the extract. The root extract significantly increased the levels of total protein and antioxidant enzymes, catalase and reduced glutathione. Improvement in lipid peroxidation was also observed. Thus it may be concluded that the *Limnanthemum cristatum* (Roxb.) Griseb possesses a protective effect against hepatotoxicity induced by CCl<sub>4</sub>. which may be attributed to the individual or combined action of phytoconstituents present in it.

**Keywords:** *Limnanthemum cristatum* (Roxb.) Griseb, silymarin, hepatoprotective activity, antioxidant enzymes, histopathology.

**Abbreviations:** *Limnanthemum cristatum* (Roxb.) Griseb, (LC).

### Introduction

The liver regulates many important metabolic functions and hepatic injury is associated with distortion of these metabolic functions. In absence of reliable liver protective drugs in modern medicine, a number of medicinal preparations from the *Ayurveda* and other Indian systems of medicine are recommended for the treatment of liver disorders [1]. Natural remedies from medicinal plants are considered to be effective and safe alternative treatment for hepatotoxicity.

*Limnanthemum cristatum* (Roxb.) Griseb, Menyanthaceae, (*Nymphoides hydrophyllum* Kuntze or *Menyanthes hydrophyllum* Lour) is found throughout India and is extensively used in Indian traditional medicine [2]. The rhizomes and roots are sold in market as 'Granthik Tagar'. Many parts of this plant find a place in the folk system of medicine such as seeds as anthelmintics, stalk and leaves for the treatment of ulcers and insect bites. This plant is used as a substitute for *Swertia chirata* and *Valleriana hardwickii* in neurological disorders and colic. The plant has also been used to treat fever, jaundice and its decoctions as a wash for parasitic skin infections [3]. According to Ayurvedic sources, this plant is widely used as hepatic tonic for many liver ailments [2]. However there are no systematic scientific reports available regarding its usefulness as a hepatoprotective agent. In view of this, the present study was undertaken to investigate the hepatoprotective activity of root extract of *Limnanthemum cristatum* (Roxb.) Griseb, (LC) against CCl<sub>4</sub> induced hepatotoxicity in male Wistar rats.

### Materials and Methods

**Chemicals:** CCl<sub>4</sub> (Merck India Ltd, Mumbai), silymarin (Micro Labs, India), thiobarbituric acid (Spectrochem Pvt. Ltd., India), trichloroacetic acid, diagnostic kits (Creast Biosystems, a division of Coral Clinical System, India) were used in the present study. All the other chemicals and reagents used were of analytical grade.

**Preparation of plant extract:** Roots of *Limnanthemum cristatum* (Roxb.) Griseb (LC), were collected from Mahavir Ayurvedic Bhandar, Mumbai, India. The plant material was identified and authenticated by Dr. Ganesh Iyer, Department of Life Science, Ramnarain Ruia College, Mumbai, India. A voucher specimen (A-006) has been preserved in our laboratory for future reference. The collected material was washed thoroughly in water and then shade dried for ten days at 35-40 °C. The dried material was pulverized in electrical grinder and passed through mesh no. 40. The obtained material was extracted in a hydroethanolic solvent (1:1) and the solvent from the extract was evaporated using rotary evaporator under reduced pressure. The dose of drug extract was prepared in water.

**Phytochemical studies:** Dragendorff's reagent was used to detect presence of alkaloids [4]. Neutral ferric chloride was used to detect phenolic compounds that appear in the form of blue spots. Folin Ciocalteu test and Fiegl's test was used to detect flavonoids and glycosides, respectively [5].

**Animals:** The experimental protocol described in the present study was approved by the Institutional Animal Ethics Committee (IAEC) of National Toxicology Centre, Pune, India, with the permission from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Healthy adult male Wistar rats (150-200 g) and female Swiss albino mice (25 g ± 5 g) were used for the study. Rats and mice were housed in small cages in a controlled environment (25 ± 2 °C, 12 h light and dark cycle) and had free

access to food and water. Animals were fed with the standard laboratory chow during the period of study.

**Acute toxicity studies:** Acute toxicity studies were performed using female Swiss Albino mice (25 g  $\pm$  5 g). The animals were fasted overnight prior to the experiment and maintained under standard laboratory conditions. LC extract was administered orally in increasing doses such as 175, 550, 1750 and 2000 mg/kg [6].

**Carbon tetrachloride (CCl<sub>4</sub>) induced hepatotoxicity in rats [7]**

A review of literature indicates a wide range of doses of CCl<sub>4</sub> being used as hepatotoxin. Hence, it was felt necessary to standardize the dose of CCl<sub>4</sub>. A pilot study was done with four doses 0.5 ml/kg, 0.75 ml/kg, 1 ml/kg, 1.5 ml/kg i.p. of a mixture of 1:1 of CCl<sub>4</sub> and olive oil. The estimation of serum SGPT level showed the increased SGPT level at the dose of 1 ml/kg i.p. which was used as dose of hepatotoxin in further studies. Similarly on the basis of a pilot study, two doses of the drug 250 mg/kg (Test 1) and 500 mg/kg (Test 2) were selected for the study. Thirty animals were classified into five groups for treatment. All groups had 6 animals in each.

- 1) Group I: Represented the control rats, that received 5 ml/kg of water per oral (p.o.) for 8 days.
- 2) Group II: 5 ml/kg of water p.o. for 8 days and CCl<sub>4</sub> 1 ml/kg, 50% v/v with olive oil, i.p on the 7<sup>th</sup> day.
- 3) Group III: LC extract at a dose of 250 mg/kg once daily, p.o. for 8 days and CCl<sub>4</sub> 1ml/kg, i.p on the 7<sup>th</sup> day .
- 4) Group IV: LC extract 500 mg/kg once daily, p.o. for 8 days and CCl<sub>4</sub> 1 ml/kg, i.p on the 7<sup>th</sup> day.
- 5) Group V: Silymarin 25 mg/kg, once daily, p.o. for 8 days and CCl<sub>4</sub> 1 ml/kg, i.p on the 7<sup>th</sup> day .

Forty eight hours after CCl<sub>4</sub> administration, the animals were sacrificed under deep urethane anesthesia. Blood was collected directly from the carotid artery and serum was separated for the assay of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), serum total bilirubin (TBil), direct bilirubin (DBil), total protein (TP) and triglycerides (STG). The livers were removed immediately, washed with ice-cold saline and a 10% homogenate prepared in phosphate buffer. The homogenate was centrifuged at 3000 rpm for 15 min at 4°C and the supernatant was used for the estimation of lipid peroxidation, catalase and reduced glutathione. Serum parameters were assayed using standard kits from Crest Biosystems, a division of Coral Clinical System, India. Lipid peroxidation was quantified by measuring the concentration of malondialdehyde (MDA) in liver homogenate using the method of Ohkawa et al [8]. The results were expressed as nmol of MDA/g wt of tissue. Reduced glutathione (GSH) was measured according to the method described by Moron et al [9]. The results were expressed as nmol of GSH/g wt of tissue. Catalase (CAT) was estimated using the method of Aebi [10] and the results were expressed as catalase enzyme activity.

**Histopathology:** The livers were removed from the animals and the tissues were fixed in 10% formalin for at least 24 hr. Then the paraffin sections were prepared (Automatic tissue processor, Auto technique) and cut into 5 mm thick sections in a rotary microtome. The sections were stained with hematoxylin-eosin dye and were studied for histopathological changes (40X), i.e. necrosis, fatty changes, ballooning degeneration, and lymphocyte infiltration.

**Statistical analysis**

The data were expressed as the mean  $\pm$  SEM of six individual observations. Results were statistically analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests using Prism 5 Software. P values  $< 0.05$  were considered to be significant.

**Results**

**Phytochemical study:** Preliminary phytochemical screening revealed the presence of alkaloids, glycosides, phenolic compounds and flavonoids.

**Acute toxicity studies:** Administration of the extract to mice did not show any sign and symptoms of toxicity and mortality upto 2000 mg/kg dose.

**Effect of extract on serum parameters:** The elevated levels of serum ALT, AST, ALP, DBil, TBil and triglycerides were significantly reduced in the rats treated with LC extract. Furthermore, the LC extract significantly increased the level of total proteins towards normalization as depicted in Table 1. Treatment with LC extract (500 mg/kg) showed significant hepatoprotective activity that was comparable to silymarin (25 mg/kg).

**Table 1.** Effects of LC extract on liver function tests in CCl<sub>4</sub> induced hepatotoxicity.

Groups	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	TBil (mg/dl)	DBil (mg/dl)	TP (g/dl)	STG (mg/dl)
Group I Normal control	38.33 $\pm 3.461^c$	88.33 $\pm 5.451^c$	287.80 $\pm 1.250^c$	0.45 $\pm 0.035^c$	0.28 $\pm 0.023^c$	7.56 $\pm 0.287^c$	61.16 $\pm 3.877^c$
Group II CCl <sub>4</sub> control 1ml/kg	140.5 $\pm 5.207$	198.6 $\pm 6.275$	616.00 $\pm 14.452$	1.62 $\pm 0.070$	1.03 $\pm 0.029$	2.98 $\pm 0.178$	109.3 $\pm 7.365$
Group III LC 250 mg/kg	89.67 $\pm 4.341^c$	163.3 $\pm 6.114^b$	464.00 $\pm 9.852^c$	1.26 $\pm 0.068^b$	0.70 $\pm 0.037^c$	5.88 $\pm 0.142^c$	75.33 $\pm 4.544^c$
Group IV LC 500 mg/kg	71.50 $\pm 5.632^c$	136.0 $\pm 8.794^c$	388.66 $\pm 2.927^c$	0.77 $\pm 0.079^c$	0.55 $\pm 0.038^c$	6.98 $\pm 0.218^c$	70.00 $\pm 3.742^c$
Group V STD 25 mg/kg	63.16 $\pm 6.194^c$	112.0 $\pm 7.465^c$	358.66 $\pm 14.252^c$	0.64 $\pm 0.054^c$	0.53 $\pm 0.034^c$	7.21 $\pm 0.204^c$	67.16 $\pm 4.542^c$

Data are expressed as mean  $\pm$  SEM, n = 6. One-way ANOVA followed by Dunnett's test when compared with CCl<sub>4</sub> control <sup>a</sup>p<0.05, <sup>b</sup>p<0.01, <sup>c</sup>p<0.001.

**Effects of extract on liver antioxidant enzymes**

Results cited in Table 2 clearly revealed the significantly increased level of MDA in Group II (CCl<sub>4</sub> control) compared to the Group I (normal control). Treatment with LC extract significantly prevented this rise in level. Levels of antioxidant enzymes, GSH and CAT were significantly increased in LC treated groups. LC (500 mg/kg) demonstrated maximum hepatoprotection.

**Table 2.** Effects of LC extract on hepatic antioxidant enzymes activity in CCl<sub>4</sub> induced hepatotoxicity

Design of Treatment	Malondialdehyde (nmol/g wt)	Reduced Glutathione (nmol/g wt)	Catalase Enzyme activity
Group I Normal control	14.11 ± 0.577 <sup>c</sup>	4.89 ± 0.133 <sup>c</sup>	74.33 ± 3.106 <sup>c</sup>
Group II CCl <sub>4</sub> control 1 ml/kg	36.55 ± 0.574	1.55 ± 0.068	41.22 ± 2.980
Group III LC 250 mg/kg	27.32 ± 0.689 <sup>c</sup>	2.92 ± 0.092 <sup>c</sup>	50.79 ± 2.183 <sup>a</sup>
Group IV LC 500 mg/kg	24.51 ± 0.941 <sup>c</sup>	3.39 ± 0.124 <sup>c</sup>	56.11 ± 1.354 <sup>c</sup>
Group V STD 25 mg/kg	22.61 ± 0.805 <sup>c</sup>	3.88 ± 0.136 <sup>c</sup>	61.52 ± 2.047 <sup>c</sup>

**3.5 Histopathology of the liver**

CCl<sub>4</sub>-induced liver injury shows large areas of centrilobular necrosis, infiltrated by mononuclear cells and macrophages; erythrophagocytosis is seen in some macrophages. The level of fatty degeneration, necrosis and vacuole formation was obvious after acute CCl<sub>4</sub> treatment. Pre-administration of extract of LC at the dose of 250 and 500 mg/kg and silymarin 25 mg/kg for 8 days could reduce the hepatic injury score of fatty degeneration and necrosis. Histological examination showed a protective effect of LC on CCl<sub>4</sub>-induced hepatotoxicity.

**Discussion**

The most encouraging finding of the study is that the LC extract can effectively prevent CCl<sub>4</sub> induced biochemical changes of the rat liver.

CCl<sub>4</sub> is a highly toxic organic solvent. It has been suggested that hepatic necrosis caused by CCl<sub>4</sub> involves bioactivation of CCl<sub>4</sub> by the microsomal cytochrome P450-dependent monooxygenase system, resulting in the formation of trichloromethyl free radical and reactive oxygen species that initiate lipid peroxidation of cell membrane and protein oxidation [11]. Loss of membrane structure and integrity because of lipid peroxidation was accompanied by an elevated level of activities of marker enzymes, AST, ALT and ALP. Accordingly, it has been shown that CCl<sub>4</sub> induces fatty liver, cell necrosis and plays a significant role in inducing triacylglycerol accumulation, depletion of reduced glutathione and loss of enzyme activity [12].

It has been well documented that both AST and ALT are among the most sensitive markers of hepatocellular injury. In liver injury, the transport function of the hepatocytes is disturbed; resulting in the leakage of plasma membrane thereby causing an increased enzymes level in serum. If injury involves organelles such as mitochondria, soluble enzymes like AST normally located there, will also be similarly released. The elevated activities of AST and ALT in serum are indicative of cellular leakage and loss of the functional integrity of cell membranes in liver [13]. Oral administration of LC to rats caused a decrease in the activity of the above enzymes, which may be a consequence of the stabilization of plasma membrane as well as repair of hepatic tissue damage caused by CCl<sub>4</sub>. This is supported by the view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and regeneration of hepatocytes [14].

The activity of serum alkaline phosphatase (ALP) was also elevated during CCl<sub>4</sub> administration. ALP is excreted normally via bile by the liver. In liver injury due to hepatotoxin, there is a defective excretion of bile by the liver which is reflected by the increased level of ALP in serum [15]. Hyperbilirubinaemia is a very sensitive test to substantiate the functional integrity of the liver and severity of necrosis which increases the binding, conjugating and excretory capacity of hepatocytes that is proportional to the erythrocyte degeneration rate [16]. Depletion of elevated bilirubin level together with the suppression of the activity of ALP in the serum of rats in group treated with LC, suggest the possibility of the LC extract being able to stabilize biliary dysfunction of rat liver during chronic injury with CCl<sub>4</sub>.

Hepatotoxicity causes a depression in total protein observed due to the defect in protein biosynthesis similar to our results. This is due to the disruption and disassociation of polyribosomes from endoplasmic reticulum following CCl<sub>4</sub> administration [17]. Administration of LC prevented this change. This may be due to the promotion of the assembly of ribosomes on endoplasmic reticulum to facilitate uninterrupted protein biosynthesis.

Treatment of rats with CCl<sub>4</sub> causes centrilobular necrosis, which results in the accumulation of fat in liver and kidney. Fat from the peripheral adipose tissue is translocated to the liver and kidney leading to its accumulation during toxicity [18]. Hepatotoxin administration produces an increase in the level of phospholipids in the liver which may be due to the decrease in mitochondrial fat oxidation. The accumulation of TG in liver of CCl<sub>4</sub>-treated rats is not due to the interference with the formation TG by the liver, but due to the inhibition or destruction of the TG secreting mechanism [19]. The observed restoration of the CCl<sub>4</sub> evoked changes in the lipid profile of serum and tissues shows the protective nature of LC.

An elevated level of MDA was observed in CCl<sub>4</sub> control group. In contrast, groups treated with LC extract showed a significant decrease in MDA levels compared to the CCl<sub>4</sub> control group. It may thus be possible that LC-supplementation was potentially effective in blunting lipid peroxidation, suggesting that LC possibly has antioxidant property to reduce toxicant induced membrane lipid peroxidation and thereby to preserve membrane structure.

Catalase (CAT) and reduced glutathione (GSH) are the most imperative antioxidants in the human body. They play a chief role in scavenging oxygen free radicals, such as superoxide anion radicals (O<sub>2</sub><sup>-</sup>·), hydroxyl radicals (·OH), supplementary free radicals (FRs) as well as singlet oxygen (<sup>1</sup>O<sub>2</sub>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and other reactive oxygen species (ROS) that are disproportionate in the human body, thereby shielding biological membranes of cells against oxidative and lipoperoxidative damages. Noticeable decreases of the levels of the above antioxidants and antioxidant enzymes in the human body can grounds variety of diseases related to the abnormal reactions of FRs.

In the present study, lower levels of CAT and GSH were observed in the CCl<sub>4</sub> control group. During hepatic injury induced by CCl<sub>4</sub>, oxygen-free radicals are generated at the site of damage and affect catalase and GSH, resulting in the loss of their activity possibly due to augmented efflux of CAT and GSH from the liver, inhibiting the biosynthesis and a binding of acetaldehyde [20]. Under oxidative stress conditions, increased radical concentrations would lead to damage to most biomolecules and, among these, antioxidant enzymes [21].

Animals have to make use of a great quantity of antioxidants in their bodies in order to scavenge the excessive ROS and FRs, and to resume and keep dynamic balance between oxidation and antioxidation, and to decrease oxidative damage in their bodies, thereby further leading to the aggravation of the oxidative stress and the oxidative damage in the liver tissue [22].

Groups treated with LC extract showed the significant amplification in the concentration of CAT and GSH as compared to CCl<sub>4</sub> control group. Thus, the capacity of *Limnanthemum cristatum* to maintain enzymatic activity could be related to its scavenging capacity for O<sub>2</sub><sup>•-</sup> and, mainly, •OH, decreasing the oxidative damage to hepatic proteins (e.g. enzymes) and thereby enhancing the enzymatic activity which may possibly detoxify the reactive oxygen species and avert CCl<sub>4</sub> induced liver toxicity.

### Conclusion

The study confirmed that prophylactic treatment with *Limnanthemum cristatum* (Roxb.) Griseb effectively inhibited the CCl<sub>4</sub> induced increase in serum biomarkers and decrease in antioxidant enzymes in rats. These results were corroborated by the histopathological findings.

However, the principal component/s responsible for this activity and the mechanism of action are currently unclear. Further phytochemical investigations using HPLC and HPTLC are required to identify, isolate and characterize the active principles responsible for the antioxidant, hepatoprotective activity and the existence of possible synergism, if any, among the compounds. Furthermore, to confirm the mechanism of action and to develop a potent hepatoprotective agent with low toxicity and better therapeutic index studies at the cellular level should be carried out.

### Acknowledgment

The authors are thankful to the National Toxicology Centre, Pune, for providing the necessary facilities and valuable suggestions for conducting the research work.

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