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Short communication

Hepatoprotective effects of *Calotropis gigantea* extract against carbon tetrachloride induced liver injury in rats

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Ethanolic extract (50 %) of stems of *Calotropis gigantea* R. Br. (*Asclepiadaceae*) at doses of 250 and 500 mg kg^{-1} were studied for hepatoprotective activity in male Wistar rats with liver damage induced using carbon tetrachloride, 2 mL kg^{-1} twice a week. The protective effect of *C. gigantea* extract was compared with the standard drug silymarin. Various biochemical parameters such as aspartate amino transferase (AST), alanine amino transferase (ALT), glutathione (GSH), lipid peroxide (LPO), superoxidedismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) were evaluated. The results revealed that the *C. gigantea* extract significantly decreased AST, ALT ($p < 0.001$) and lipid peroxide ($p < 0.01$) levels. The antioxidant parameters GSH, GPx, SOD and catalase levels were increased considerably compared to their levels in groups not treated with *C. gigantea* extract.

Keywords: *Calotropis gigantea* (*Asclepiadaceae*), hepatoprotective activity, serum enzymes, antioxidants

Reactive oxygen species (ROS) are inevitably generated, due to the incomplete reduction of O_2 in electron transfer reactions, as byproducts of biological reactions. When ROS production is greater than the detoxification capacity of the cell, excessively generated ROS causes extensive damage to DNA, proteins and lipids and acts as a mediator of pro-inflammatory and carcinogenic events (1). To avoid redox imbalance and oxidative damage, aerobic organisms possess efficient biochemical defense systems such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (2), though it cannot completely protect them from severe oxidative stress. In this context, many scientists have tried to obtain dietary antioxidants such as ascorbate, tocopherol and carotenoids from fruits and vegetables, because they could help protect cells from cellular damage caused by oxidative stress.

Several experimental animal models of hepatotoxicity have been developed to investigate the toxicological mechanisms between environmental contaminants and liver.

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Thus, the animal model of acute liver injury induced by carbon tetrachloride (CCl_4) has been utilized to screen hepatoprotective compounds (3), because trichloromethyl free radicals (CCl_3) as metabolites of CCl_4 initiate a lipid peroxidation process in cellular membranes, eventually leading to various liver pathological processes, such as fibrogenesis, cirrhosis or steatosis (4).

Calotropis gigantea R. Br. (*Asclepiadaceae*), a wildly growing plant, has been reported to possess a number of medicinal properties and is used in toothache and earache, sprain, anxiety, pain, epilepsy and in mental disorders (5). Aerial parts of the plant have been reported to possess anti-diarrheal properties (6) and flowers are known for their analgesic activity (7). The roots of the plant have shown CNS activity (5) as well as pregnancy interceptive properties (8).

The stem bark of *C. gigantea* yields resin and wax. The wax contains β-amyrin and its isovalerate, α and β-calotropeols, mixture of tetracyclic triterpene, traces of sterols, C_{31} and C_{33} hydrocarbons, fatty acids and giganteol. The stem of *C. gigantea* yields latex. The latex contains cardiac glycosides, calotropin, uscharin, calotoxin, uscharidin and gigantin. The proteolytic enzyme calotropain has been isolated from the latex. Calotropain has marked anti-blood coagulating activity. The latex consists of calotropin D_I and D_{II} and calotropain F_I and F_{II} and an enzyme with invertase activity. It is a promising anti--inflammatory agent (9).

Phytochemical investigations of the leaves of *C. gigantea* have shown the presence of taraxasterly acetate, pinoresinol, medioresinol, uzarigenin, calotropin, calactin, calacitnic acid, calacitnic acid methyl ester, 19-carboxyl-calacitnic methyl ester, drummondol, 15β-hydroxycalotrin, the C_{11} bicylic lactone norisopenoid, the rare diphenyl furfuran lignan, salicifoliol and 19-nor- and 18,20-epoxy-cardenolides (10).

The aim of the present study is to evaluate the hepatoprotective activity of the 50 % ethanolic extract of *Calotropis gigantea* stems and to compare its efficacy with silymarin.

EXPERIMENTAL

Plant material

The stems of *Calotropis gigantea* (*Asclepiadaceae*) were procured locally from Lucknow district of Uttar Pradesh in India and were identified at the Hygia Institute of Pharmaceutical Education and Research, Lucknow (India). Voucher specimens are kept in the herbarium (HIPER/07/12) of the Institute for further references.

Stems were washed with tap water, chopped into pieces and dried in shade. Dried stem pieces were ground to coarse powder and stored in an airtight container. The dried stems were extracted (250 g) with ethyl alcohol (50 %, *V/V*) in a Soxhlet extractor for 18–20 h. The extract was concentrated to dryness under reduced pressure and controlled temperature (40–50 °C).

The extract thus obtained was preserved in a desiccator to prevent degradation by moisture. For pharmacological studies, the *C. gigantea* extract was suspended in doubly distilled water containing carboxymethyl cellulose (1 %, *m/V*, CMC). Preliminary phyto-

chemical screening was carried out on the *C. gigantea* extract to assess the presence of alkaloids, glycosides, saponins, flavanoids and steroids.

Drugs and chemicals

All the drugs and chemicals used in the study were of analytical grade. Carbon tetrachloride was obtained from Merck Limited, India. Silymarin was obtained from Ranbaxy Laboratories Limited, India. The chemicals used for evaluation of oxidative stress parameters were obtained from Sisco Research Laboratories, India. Kits used for the estimation of serum aspartate and alanine transaminase (AST and ALT) levels were purchased from Centronic GmbH, Germany. Folin-Ciocalteu reagent was purchased from Sigma Chemicals (USA).

Determination of total phenolics

One hundred milligrams of the *C. gigantea* extract was dissolved in 250 mL of methanol/water (60:40, *V/V*, 0.3 % HCl) and filtered through a 0.45-μm Millipore filter. To 100 μL of filtrate, 100 μL of Folin-Ciocalteu reagent (50 %, *V/V*) and 2.0 mL of sodium carbonate (2 %, *m/V*) were added and mixed completely. After 2 hours, the absorbance of the solution was measured at 750 nm. Quantification was based on the standard curve of gallic acid (0–1.0 mg mL^{-1}) dissolved in methanol/water (60:40, *V/V*, 0.3 % HCl). Phenolic content was expressed as milligrams per gram of gallic acid equivalent (GAE).

Experimental animals

Studies were carried out using male Wistar albino rats (180–220 g). They were obtained from the animal house of the Hygia Institute of Pharmaceutical Education and Research, Lucknow, India. The animals were grouped and housed in polyacrylic cages (38 × 23 × 10 cm) with not more than six animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2 °C) with light and dark cycles of 12 and 12 h, respectively. They were allowed free access to the standard dry pellet diet and water *ad libitum*. All procedures described were reviewed and approved by the Institutional Animal Ethical Committee.

Hepatoprotective study

The rats were divided into five groups of six animals each. Group I, normal control, was given 1 mL normal saline orally once daily. Groups II through V received subcutaneous injections of CCl_4 in olive oil (1:1) at a dose of 2 mL kg^{-1} twice a week to induce hepatotoxicity. Group II served as CCl_4 control. Groups III and IV received *C. gigantea* extract at doses of 250 and 500 mg kg^{-1}. Group V received silymarin at a dose of 100 mg kg^{-1}. In the treatment groups, *C. gigantea* extract and silymarin were administered orally once daily, starting 2 h before the CCl_4 injection. After 1 week of treatment, the rats were sacrificed, blood was collected and serum was separated.

Biochemical analyses

The AST and ALT levels in the serum were estimated using commercially available kits.

The reduced glutathione (GSH) level in the liver was determined according to the method of Ellman (11); hepatic superoxide dismutase (SOD) activity by the method of Kakkar *et al*. (12), glutathione peroxidase (GPx) by the method of Paglia and Valentine (13) and catalase by the method of Aebi (14).

The hepatic TBARS level, an index of malonyldialdehyde (MDA) production, was determined by the method of Ohkawa *et al.* (15).

Histopathological analysis of liver

For histological studies, liver tissues were fixed with 10 % phosphate-buffered neutral formalin, dehydrated in graded (50–100 %) alcohol and embedded in paraffin. Thin sections were cut and stained with hematoxylin and eosin stain for microscopic assessment. The initial examination was qualitative, with the purpose of determining histopathological lesions in liver tissue.

Statistical analysis

The values are expressed as mean ± SEM of six observations. The results obtained were statistically analyzed by Student's *t*-test.

RESULTS AND DISCUSSION

The preliminary phytochemical screening of *C. gigantea* extract indicated the presence of triterpenoids, steroids, flavonoids and glycosides. The extract was found to contain 330.0 ± 13.6 mg g^{-1} total polyphenolics expressed as GAE (milligrams per gram of GAE).

The effect of *C. gigantea* extract on AST and ALT levels in rats with CCl_4 induced hepatotoxicity is summarized in Figure 1. There was a significant ($p < 0.001$) increase in

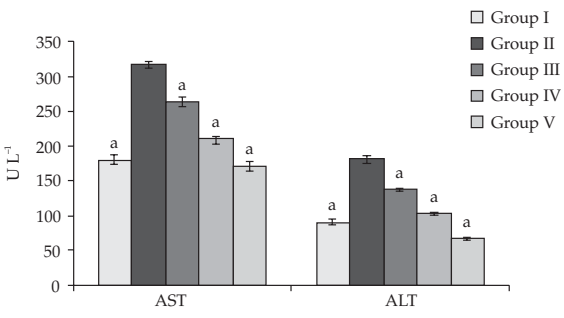

Fig. 1. Effect of *C. gigantea* on serum AST and ALT levels in rats with CCl_4-induced hepatotoxicity. Values represent mean ± SEM of six animals in each group ($^a p < 0.001$ *vs*. group II).

the AST and ALT levels in rats treated with CCl_4 alone compared to normal control. The AST levels significantly ($p < 0.001$) increased to 317.00 U L^{-1} in CCl_4-treated rats and was considerably reduced on treatment with *C. gigantea* extract at doses of 250 mg kg^{-1} (263.17 U L^{-1}, $p < 0.001$) and 500 mg kg^{-1} (210.19 U L^{-1}, $p < 0.001$). The ALT levels of the rats treated with CCl_4 were found to be increased (181.13 U L^{-1}) and there was a significant ($p < 0.001$) reduction to 137.2 U L^{-1} and 103.13 U L^{-1} by *C. gigantea* extract at 250 mg kg^{-1} and 500 mg kg^{-1} dose respectively. The AST and ALT levels were significantly ($p < 0.001$) reduced to 171.33 and 66.81 UL^{-1}, repectively, by administration of silymarin at a 100 mg kg^{-1} dose.

The effects of *C. gigantea* extract on GSH, TBARS, SOD, GPx and catalase in rats with CCl_4-induced hepatotoxicity are summarized in Table I. For example, there was a marked decrease in GSH level in rats treated with CCl_4, *i.e.* 2.63 mg g^{-1} as compared to 7.51 mg g^{-1} ($p < 0.001$) in normal control rats. The GSH level was significantly increased to 3.71 ($p < 0.01$), 4.15 ($p < 0.001$) and 5.37 ($p < 0.001$) by the treatment with *C. gigantea* extract at 250 mg kg^{-1}, 500 mg kg^{-1} and silymarin at 100 mg kg^{-1} doses, respectively. The data obtained in the present study (Table I) clearly shows an increase in the MDA levels of rats treated with CCl_4 suggesting enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. The increase in TBARS levels (37.13 mmol g^{-1} compared to 8.83 mmol g^{-1} in normal control) was significantly reduced by the treatment with *C. gigantea* extract at doses of 250 mg kg^{-1} (31.11 mmol g^{-1}, $p < 0.01$), 500 mg kg^{-1} (26.11 mmol g^{-1}, $p < 0.001$) and by the treatment with silymarin at dose of 100 mg kg^{-1} (7.78 mmol g^{-1}, $p < 0.001$).

The SOD levels in normal control rats were 148.11 U mg $protein^{-1}$ which was reduced to 69.23 U mg $protein^{-1}$ in rats treated with CCl_4. The level of SOD was increased again to 77.12 U mg $protein^{-1}$ and 108.43 U mg $protein^{-1}$ ($p < 0.001$) by the administration of *C. gigantea* extract at dose of 250 mg kg^{-1} and 500 mg kg^{-1}, respectively. Silymarin at 100 mg kg^{-1} dose increased the SOD levels to 120.37 U mg $protein^{-1}$ ($p < 0.001$).

Table I. Effect of C. gigantea *extract on various parameters of oxidative stress in rats with CCl_4-induced hepatotoxicity*

Group	GSH (mg g^{-1})	TBARS (mmol g^{-1})	SOD (U mg $protein^{-1}$)	GPx (U mg $protein^{-1}$)	Catalase (U mg $protein^{-1}$)
Group I	7.51 ± 0.17^{b}	8.83 ± 0.67^{b}	148.11 ± 0.61^{b}	51.14 ± 5.13^{b}	5.18 ± 0.38^{b}
Group II	$2.63 \pm 0.27^{a,b}$	$37.13 \pm 0.88^{a,b}$	69.23 ± 5.91^{b}	$20.11 \pm 1.71^{a,b}$	$2.91 \pm 0.13^{a,b}$
Group III	3.71 ± 0.24^{a}	31.11 ± 2.16^{a}	77.12 ± 2.79	27.91 ± 2.97^{a}	3.11 ± 0.21
Group IV	4.15 ± 0.16^{b}	26.11 ± 1.59^{b}	108.43 ± 3.91^{b}	32.93 ± 0.98^{b}	4.63 ± 0.37^{a}
Group V	5.37 ± 0.18^{b}	7.78 ± 0.58^{b}	120.37 ± 6.95^{b}	47.72 ± 0.56^{b}	4.97 ± 0.11^{b}

Values represent mean ± SEM of six animals in each group.
Group I – normal control, group II – CCl_4 (2 mL kg^{-1}) twice a week, group III – *C. gigantea* extract (250 mg kg^{-1}) + CCl_4 (2 mL kg^{-1}) twice a week, group IV – *C. gigantea* extract (500 mg kg^{-1}) + CCl_4 (2 mL kg^{-1}) twice a week, group V – silymarin (100 mg kg^{-1}) + CCl_4 (2 mL kg^{-1}) twice a week.
Significant difference between groups I, III–V and group II: $^{a}p < 0.01$, $^{b}p < 0.001$.

The level of GPx in rats treated with CCl_4 was 20.11 U mg protein^{-1} (a reduction from 31.03 U mg protein^{-1} compared to normal control). The levels of GPx increased again to 27.91 ($p < 0.01$) and 32.93 ($p < 0.001$) U mg protein^{-1} in rats treated with *C. gigantea* extract of 250 mg kg^{-1} and 500 mg kg^{-1}, respectively. Silymarin produced a significant ($p < 0.001$) increase to 47.72 U mg protein^{-1} in GPx levels at 100 mg kg^{-1} dose.

Administration of *C. gigantea* extract increased the CAT level in CCl_4 treated rats with induced liver damage, thus preventing accumulation of excessive free radicals and protecting the liver from CCl_4 intoxication (Table 1). The level of catalase (2.91 U mg protein^{-1}) was significantly increased by the administration of *C. gigantea* extract (500 mg kg^{-1} to 4.63 U mg protein^{-1} ($p < 0.01$) and by silymarin (100 mg kg^{-1}) to a 4.97 U mg protein^{-1} value ($p < 0.001$).

Histological observations basically supported the results obtained from serum enzyme assays. The liver of CCl_4-intoxicated rats showed massive fatty changes, gross necrosis and broad infiltration of lymphocytes and Kupffer cells around the central vein and loss of cellular boundaries. Histopathological observations of the liver of rats pretreated with *C. gigantea* extract and subsequently given CCl_4 showed a more or less normal architecture of the liver, having reversed to a large extent the hepatic lesions produced by the toxin, almost comparable to the normal control and the silymarin group (Figs. 2a-d).

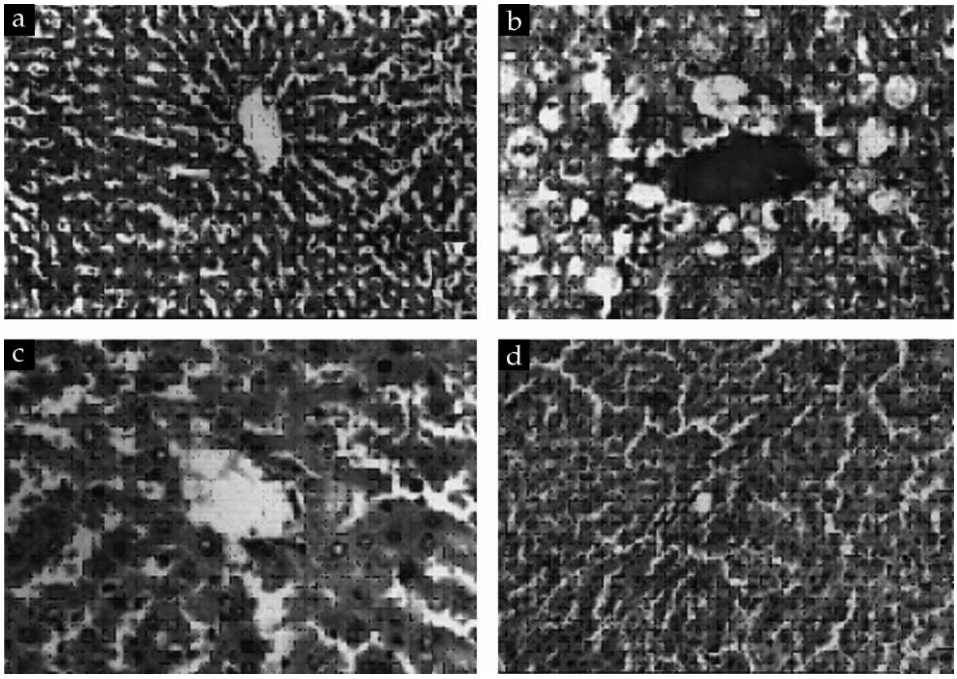

Fig. 2. Section of the liver of a) normal control rat, b) a rat treated with carbon tetrachloride, c) a rat treated with *C. gigantea* extract (500 mg kg^{-1}) + CCl_4 (2 mL kg^{-1}) twice a week, d) a rat treated with silymarin (100 mg kg^{-1}) + CCl_4 (2 mL kg^{-1}) twice a week.

CONCLUSIONS

Based on the results obtained, it may be concluded that the *Calotropis gigantea* stem extract has a significant effect on liver injuries as well as on oxidative stress, resulting in reduced lipid peroxidation and improved serum biochemical parameters such as AST and ALT. The reduced levels of parameters of SOD, CAT, GSH and GPx in CCl_4-treated rats were significantly increased by treatment with *C. gigantea* extract.

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S A Ž E T A K

Hepatoprotektivno djelovanje ekstrakta biljke *Calotropis gigantea* na oštećenje jetre štakora tetraklormetanom

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Etanolni ekstrakt (50 %) stabljika biljke *Calotropis gigantea* R. Br. (*Asclepiadaceae*) u dozi 250 i 500 mg kg^{-1} testiran je na hepatoprotektivno djelovanje na oštećenje jetre mužjaka Wistar štakora inducirano tetraklormetanom, 2 mL kg^{-1} dva puta tjedno. Zaštitni učinak ekstrakta biljke *C. gigantea* uspoređivan je sa standarnim lijekom silimarinom. Evaluirani su različiti biokemijski parametri kao što su aspartat amino transferaza (AST), alanin amino transferaza (ALT), glutation (GSH), lipidni peroksidi (LPO), superoksid-dismutaza (SOD), glutation peroksidaze (GPx) i katalaza (CAT). Rezultati ukazuju da ekstrakt biljke *C. gigantea* značajno smanjuje koncentracije AST, ALT ($p < 0.001$) i lipidnih peroksida ($p < 0.01$). Koncentracije antioksidativnih parametara GSH, GPx, SOD i katalaze bile su značajno povišene u usporedbi s njihovim koncentracijama u skupinama koje nisu tretirane ekstraktom biljke *C. gigantea*.

Ključne riječi: *Calotropis gigantea* (*Asclepiadaceae*), hepatoprotektivno djelovanje, enzimi seruma, antioksidansi

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