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Anti-Hepatotoxic Property of Roots and Leaves of *Chassalia curviflora* (Wall.) Thwaites against CCl_4 Induced Liver Damage in Rats

G. Rajeswari Gopal[1*], G.I. Anuja[2], P.G. Latha[2], A. Jayakumaran Nair[1], A. Gangaprasad[4], P.R. Sudhakaran[1], D. Muraleedharan[3], G.M. Nair[1]

[1]*Inter University Centre for Genomics and Gene Technology, University of Kerala, Kariavattom-695581, Kerala, India*
[2]*Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram-695 562, Kerala, India*
[3]*Salim Ali Centre for Ornithology and Natural History, Coimbatore-641108, Tamil Nadu, India*
[4]*Department of Botany, University of Kerala, Kariavattom-695581, Kerala, India*

ABSTRACT

In the present study the roots and leaf ethanolic extracts of *Chassalia curviflora* were screened for their anti hepatotoxic and anti oxidant effects in experimentally induced liver injury by carbon tetra chloride. Liver serum marker enzymes as well as antioxidant enzymes mainly superoxide dismutase, catalase and glutathione levels were determined. The plant root and leaf ethanolic extracts at 50, 100, 200 mg concentrations significantly (** $P \leq 0.05$) reduced the elevated serum hepato specific enzyme [serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), serum alkaline phosphatase (SAKP), serum bilirubin (SB)] and cholesterol levels induced by CCl_4. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and non enzymatic reduced glutathione (GSH) levels were increased whereas malondialdehyde (MDA) level was reduced by the extracts. Compared to the leaf extract, root extract significantly reduced serum hepato parameters and increased the levels of anti-oxidant enzymes in a dose dependent manner. The results were comparable to that of silymarin, reference drug used for the study. The histopathological studies also supported the biochemical findings. Acute toxicity studies revealed that it is safe for pharmacological uses upto 2000mg/kg. The present study scientifically validated the traditional use of plant root in hepatoprotection.

Keywords: Anti-hepatotoxic, *Chassalia curviflora*, CCl_4 and silymarin.

INTRODUCTION

Chassalia curviflora (Wall.) Thwaites (syn. *Psychotria curviflora* (Wall.)) belonging to the family Rubiaceae, is an erect shrub with white or tinged pinkish flower and shiny black fruits that grows widely in Western Ghats of India. It is distributed from India to South China and Philippines. The plant is commonly called curved flower woody chassalia and Vellakurinji, Yamari or Mundanchedi in Malayalam. In India the plant is Meghalaya,

Maharashtra, Karnataka, Andaman and Western Ghats. Decoction of the roots is taken for rheumatism, pneumonia, malaria, cough and phlegm. [1-2] Root and leaves are applied topically to cure wounds, ulcers and also to treat headache. [1] Chakma tribes of Bangladesh make use of crushed leaves to the wounds for treating snake and insect bites. [3] It is widely used by Kani tribes of Agasthiyamalai, Kerala, as an effective

***Corresponding author: Ms. G. Rajeswari Gopal,**
Inter University Centre for Genomics and Gene Technology, University of Kerala, Kariavattom-695581, Kerala, India; **E-mail:** rajeswarigopal@gmail.com
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medicine for the treatment of jaundice. [4] The anti-inflammatory and analgesic roles of both the root and leaf ethanolic extracts in experimental animals were reported. [5]

Liver, being the largest organ in the human body plays a major role in regulating metabolism, secretion and storage. It is involved in almost all of the biochemical pathways like protein, carbohydrate and fat metabolism, secretion of bile, detoxification and vitamin storage. [6] A healthy liver is needed to maintain homeostasis. Liver injury is caused by different agents such as chemicals, alcohols, viruses and auto immune diseases. About 25,000 deaths occur worldwide every year due to several liver disorders [7] which includes hepatitis, hepatic steatosis, jaundice and cirrhosis. There is hardly any drug in modern medicine that can offer protection to the liver from damage and stimulate liver function and also help in regeneration process. [8]

Plants which are used traditionally for liver dysfunction may provide useful hepatoprotective compounds for the progress of new pharmaceuticals or can be an adjunct to present therapy. [9] In folklore medicine many herbal formulations are available for liver disorders. In this vision the present study was to investigate the anti- hepatotoxic and anti oxidant effects of ethanolic extracts of *C. curviflora* roots and leaves.

MATERIALS AND METHODS

Plant material and Preparation of plant extract

C. curviflora leaves and roots were collected from the medicinal plant garden of University of Kerala, Kariavattom campus, Thiruvananthapuram. The plant was identified by the Curator, University of Kerala and a voucher specimen was deposited at the Jawaharlal Nehru Tropical Botanic Garden and Research Institute, (JNTBGRI), Palode (TBGT 57053 23/9/11). Fifty grams of the powdered leaves and roots were extracted separately with 500mL of 95% ethanol with continuous stirring at room temperature. The aliquot was gradually concentrated in vacuum and allowed to dry. The root and leaf extract percentage yield was calculated and were labelled as Chr and Chl respectively. The samples were stored at -4°C until use.

Phytochemical Screening

Phytochemical screenings of crude ethanolic extracts were done according to the standard protocol to test the presence of chemical constituents. [10] Flavonoid with the use of Mg and HCl, tannins with ferric chloride and saponins with the ability to produce stable foam and alkaloids with Dragendroff's reagent, picric acid and Mayer's reagent.

Experimental Animals

Wistar rats (150-200 g) of either sex were obtained from the animal house of JNTBGRI. They were grouped and housed in poly-acrylic cages (six animals per cage) and maintained under standard laboratory conditions (temperature 24-28°C, relative humidity 60-70% and 12 hours dark- light cycles). They were fed commercial rat feed (Lipton India Ltd, Mumbai, India) and boiled water, *ad libitum*. All animal experiments were carried out according to NIH guidelines, after getting the approval of the JNTBGRI's Animal Ethics Committee.

In vivo Pharmacological studies- CCl_4 induced liver toxicity study in Wistar rats [11]

Wistar rats were divided into nine groups, each containing six rats. Animals of group 1 (normal control) were treated with a single daily dose of 0.5% tween-80 (1 ml, *p.o.*) on all 5 days and olive oil (1 ml/kg, *s.c.*) on days 2 and 3. Animals of group 2 (CCl_4 control) were treated with a single daily dose of 0.5% tween-80 (1 ml, *p.o.*) for 5 days and then CCl_4 was administered (2 ml/kg, *s.c.*) diluted in olive oil (1:1), on days 2 and 3. Animal of group 3, 4 and 5 were treated with Chr (50, 100 and 200 mg/kg, *p.o.*) for 5 days; then a single dose of CCl_4 (2 ml/kg, *s.c.*) diluted in olive oil (1:1), on days 2 and 3, 30 min after drug treatment. Animal of group 6, 7 and 8 were treated with Chl (50, 100 and 200 mg/kg, *p.o.*) for 5 days; then a single dose of CCl_4 (2 ml/kg, *s.c.*) diluted in olive oil (1:1), on days 2 and 3, 30 min after drug treatment. Animals of group 9 were treated with silymarin (100 mg/kg, *p.o.*) for 5 days; on days 2 and 3, a single dose of CCl_4 (2 ml/kg, *s.c.*) diluted in olive oil (1:1), 30 min after silymarin administration.

Assessment of Liver function

Serum biochemical parameters-On the 5th day of the CCl_4 induced hepatotoxicity study all the animals were sacrificed by carbon dioxide inhalation. Blood samples were collected for evaluating the serum biochemical parameters glutamate pyruvate transaminase (SGPT) [12] glutamate oxaloacetate transaminase (SGOT) [12] alkaline phosphatase (SAKP) [13], bilirubin (SB) [14] and cholesterol. [15]

Assessment of Liver tissue parameters (antioxidant status of liver)

Liver samples were also collected and stored in liquid nitrogen for assessment of tissue parameters malondialdehyde (MDA) [16], reduced Glutathione (GSH) [17], superoxide dismutase (SOD) [18], catalase (CAT) activity [19] and total protein. [20]

Histopathological Studies

After blood draining, liver samples were excised from the control and treated groups of animals and washed with normal saline separately. They were fixed in 10% buffered formalin. The formalin-fixed liver samples were stained with haematoxylin-eosin for microscopic observations of the liver histological architecture, on a Carl Zeiss microscope with photographic attachment.

Behavioral and Toxic effects [5]

Fourteen groups of 10 mice were administered orally 50,100, 200, 500, 1000, 1500, 2000 mg/kg of Chr and Chl respectively maintaining appropriate controls. All the animals were observed continuously for the first 3 h and then 1 h intermittently up to 24 h for behavioral changes. The animals were observed for post treatment toxic symptoms daily for 7 days after treatment.

Statistical Analysis

The analysis was carried out using the Students't'-test. [21] Results were reported as mean ± S.D and the't' test was used to evaluate difference between groups with $P \leq 0.05$, considered as significant.

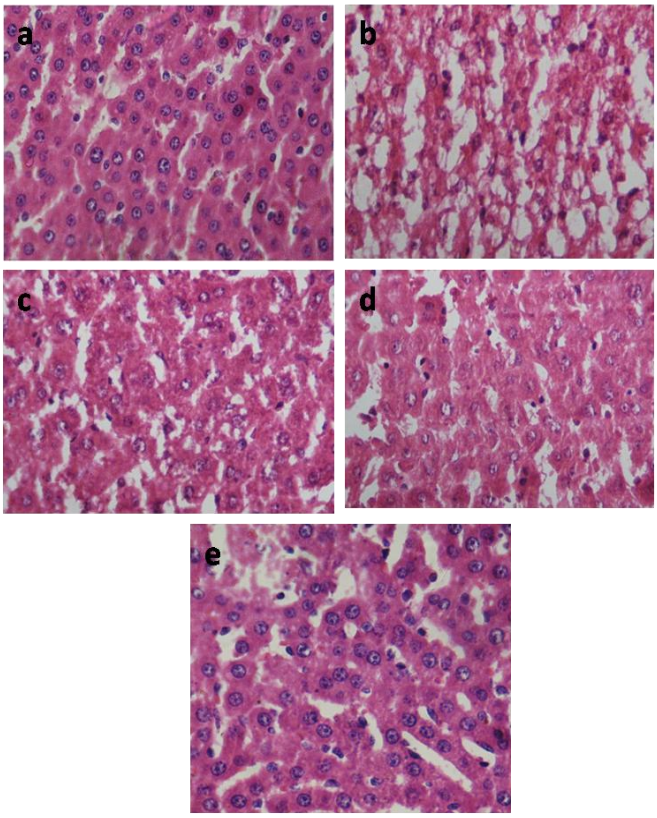

Fig. 1: Micrograph of liver of rats: (a) Liver of normal control rat showing hepatic cells with normal nuclei and cytoplasm. (b) Section of CCl_4 treated rat liver, showing Kupffer cells, and extensive vacuolization (c) Section of Chr (200 mg / kg) + CCl_4 treated rat liver showing marked improvement in histological architecture over CCl_4 control group (d) Section of Chl (200 mg / kg) + CCl_4 treated rat liver showing almost normal histological architecture (e) Section of Silymarin (100mg / kg) + CCl_4 treated rat liver showing regeneration of hepatic cells with normal architecture.

Table 1: Preliminary phytochemical screening of root and leaves of *C. curviflora*

Phytochemicals	Ethanolic root extract (Chr)	Ethanolic leaf extract (Chl)
Alkaloids	+	+
Sterols	+	+
Terpenoids	+	+
Saponins	+	+
Tannins	+	+
Flavonoids	+	-
Amino acids	+	+
Resins	+	-
Phenols	+	-
Cardiac glycosides	+	+
Reducing Sugar	+	+
Quinines	+	-
Steroids	+	+

'+' sign indicates the presence of phytochemical
'-' sign indicates the absence of phytochemical

RESULTS

Phytochemical Screening

The Chr and Chl ethanolic extracts were screened for the presence of various phytochemicals. Compared to leaf, root ethanolic extract possess almost all the phytochemicals. In both Chr and Chl, the presence of alkaloids was more. The results are documented in Table 1. The percentage yield was 0.65% for Chr and 0.8 % for Chl.

CCl_4 Induced Hepatotoxicity

Rats treated with carbon tetrachloride (CCl_4) developed significant hepatic damage. There was an elevation in serum liver specific enzyme levels and alteration in different liver parameters. *C. curviflora* root and leaf extracts provided significant protection against CCl_4 induced increase in serum enzyme levels in a dose responsive manner.

The degree of protection was observed maximally with the highest dose of the extract. Treatment with 200 mg/kg of both root (Chr) and leaf (Chl) extracts could significantly reduce the serum levels of SGOT and serum bilirubin to near comparable level produced by the standard drug (Table 2).

Oral administration of Chr (200 mg/kg) could bring down the level of hepato specific biochemical parameters and the values were significantly comparable with the effect produced by the standard drug silymarin. Hepatoprotective efficacy of root extracts was evident especially as it could bring down the level of SGPT, SAKP and cholesterol to levels almost on par with the treatment of silymarin. The leaf ethanolic extract was also found to considerably reduce the hepatotoxicity induced by CCl_4 treatment as observed by the reduced levels of biochemical parameters.

The effect of oral administration of Chr, Chl and reference drug on serum levels of SGOT, SGPT, SAKP, serum bilirubin and cholesterol which serve as markers of hepatotoxicity in normal and CCl_4 treated rats are presented in Table 2. The *C. curviflora* root and leaf extracts afforded a significant protection against CCl_4 induced increase in serum enzyme levels in a dose responsive manner. The degree of protection was observed maximally with the highest dose of the extracts.

Liver Tissue parameter study

In liver serum, GSH, SOD and CAT levels were considerably reduced and MDA level was increased in CCl_4 treated rats compared to normal rats. A significant rise in these serum enzymes was observed upon treatment with Chr and Chl (Table 3). Significant protective activities for GSH, SOD and CAT was achieved in silymarin treated liver as well and a near normal level for these antioxidant enzymes could be reached on treatment with *C. curviflora* root extracts at 200 mg/kg dose.

The activities of enzymatic and non enzymatic antioxidants were presented in Table 3. In liver serum GSH, SOD and catalase levels were considerably reduced and MDA level was increased in CCl_4 induced rats compared to normal rats. However treatment with Chr and Chl showed significant protective activities for GSH, SOD and catalase at all the three doses.

Table 2: Effect of *C. curviflora* root (Chr) and leaf ethanolic extracts (Chl) and silymarin on serum enzyme levels of rats after carbon tetrachloride (CCl_4) administration

Groups	SGOT (IU/L)	SGPT (IU/L)	SAKP (KA Units)	SB (mg/dL)	Cholesterol (mg/dL)
Normal control	44.06 ± 1.73	46.72 ± 3.31	54.72 ± 1.54	0.27 ± 0.03	51.45 ± 1.55
CCl_4 control (2 ml/kg)	177.55 ± 5.12	291.38 ± 4.99	165.32 ± 5.52	2.23 ± 0.89	83.75 ± 1.74
CCl_4 (2 ml/kg) + Chr (50 mg/kg)	91.25 ± 2.56	134.82 ± 4.29	77.78 ± 4.71	1.73 ± 0.78	75.05 ± 3.42
CCl_4 (2 ml/kg) + Chr (100 mg/kg)	81.88 ± 1.61**	117.72 ± 5.73**	70.71 ± 300**	1.20 ± 0.18**	68.45 ± 4.31**
CCl_4 (2 ml/kg) + Chr (200 mg/kg)	73.81 ± 3.32**	108.30 ± 4.52**	68.52 ± 2.93**	0.87 ± 0.17**	61.62 ± 3.16**
CCl_4 (2 ml/kg) + Chl (50 mg/kg)	99.83 ± 3.60	143.39 ± 2.78	85.12 ± 4.62	2.09 ± 0.29	77.83 ± 3.67
CCl_4 (2 ml/kg) + Chl (100 mg/kg)	86.54 ± 2.68	125.20 ± 2.19	78.38 ± 1.86	1.55 ± 0.15	73.97 ± 2.39
CCl_4 (2 ml/kg) + Chl (200 mg/kg)	78.59 ± 2.85**	115.39 ± 1.88**	73.69 ± 2.00**	1.01 ± 0.19**	69.64 ± 2.96**
CCl_4 (2 ml/kg.) + Silymarin (100 mg/kg)	58.79 ± 3.87**	92.54 ± 2.28**	62.58 ± 5.37**	0.47 ± 0.36**	55.52 ± 3.07**

Values are the mean ± S.D, n = 6 Students `t` test $^{**}P \leq 0.05$, compared to CCl_4 control

Table 3: Effect of *C. curviflora* root and leaf ethanolic extracts (Chr and Chl) and silymarin on liver tissue parameters of rats after carbon tetrachloride (CCl_4) administration

Groups	MDA (nmol/mg wet wt of liver)	GSH (nmol/mg protein)	SOD (Unit/mg protein)	CAT (Unit/mg protein)
Normal control	0.57 ± 0.06	0.67 ± 0.03	5.46 ± 0.16	3.07 ± 0.29
CCl_4 control (2 ml/kg)	1.24 ± 0.39	0.20 ± 0.06	2.21 ± 0.49	0.64 ± 0.03
CCl_4 (2 ml/kg) + Chr (50 mg/kg)	0.94 ± 0.04	0.31 ± 0.01	2.95 ± 0.32	1.31 ± 0.29
CCl_4 (2 ml/kg) + Chr (100 mg/kg)	0.82 ± 0.12	0.38 ± 0.02	3.60 ± 0.36	1.79 ± 0.26
CCl_4 (2 ml/kg) + Chr (200 mg/kg)	0.73 ± 0.02**	0.49 ± 0.03**	4.29 ± 0.35**	2.44 ± 0.32**
CCl_4 (2 ml/kg) + Chl (50 mg/kg)	1.02 ± 0.12	0.25 ± 0.28	2.60 ± 0.21	0.97 ± 0.05
CCl_4 (2 ml/kg) + Chl (100 mg/kg)	0.92 ± 0.27	0.36 ± 0.28	3.66 ± 0.20	1.68 ± 0.25
CCl_4 (2 ml/kg) + Chl (200 mg/kg)	0.83 ± 0.10**	0.45 ± 0.02**	3.95 ± 0.13**	1.88 ± 0.44**
CCl_4 (2 ml/kg) + Silymarin (100 mg/kg)	0.64 ± 0.04**	0.67 ± 0.05**	4.63 ± 0.35**	2.65 ± 0.22**

Values are the mean ± S.D, n = 6 Students `t` test $^{**}P \leq 0.05$, compared to CCl_4 control

Histopathological Study

The results of histopathological studies provided supportive evidence for biochemical analysis. Histology of liver section (Fig. 1) of normal control animals exhibited normal hepatic cells each with well defined cytoplasm, prominent nucleus and nucleolus. Whereas that of CCl_4 intoxicated animals showed total loss of hepatic architecture with centrilobular hepatic necrosis, fatty changes, vacuolization, and congestion of sinusoids, kupffer cells hyperplasia, crowding of central vein and apoptosis. The histopathological observations of the liver of rats pretreated with Chr and Chl and subsequently given CCl_4 showed a more or less normal histological architecture of the liver which is comparable to the normal control and sylimarin groups.

Behavioral and Toxicity study

In the behavioral and toxicity studies, the mice did not show any gross behavioral changes and no mortality occurred within 24 hour with the four doses of Chr and Chl tested. The LD_{50} of Chr and Chl was found to be greater than 2000 mg/kg.

DISCUSSION

The reports on the traditional use of *Chassalia curviflora* for treatment of jaundice by the indigenous tribe of Kani [22-23], necessitates the validation of hepatoprotective efficacy of the plant extract to be used as a lead for the discovery of plant based hepatoprotective drugs. Since management of liver disorders by a simple and precise herbal drug is still an intriguing problem, there is an ever increasing need for safe hepatoprotective herbal agent [24], the use of which is the need of the hour considering the increased side effects being caused by the synthetic drugs. Moreover, the traditional use of *C. curviflora* for treatment of jaundice has still not been scientifically studied. [25]

The presence of several phytochemicals in plant extracts account for its usefulness as medicinal plant. The root and leaf ethanolic extract of the plant are reported to possess anti-inflammatory and analgesic activity. [5] Liver damage induced by carbon tetra chloride (CCl_4) is commonly used as a model for the screening of hepatoprotective drugs. The hepatotoxicity of CCl_4 is mainly due to the presence of active metabolite trichloromethyl radical ($CCl_3^{\bullet}$). Carbon tetra chloride is metabolically activated by cytochrome P-450 dependent oxidase in the endoplasmic reticulum to form trichloromethyl free radical ($CCl_3^{\bullet}$) which disrupts the structure and functions of cellular lipids and proteins in the membranes of cellular organelles. [26-29] Lipid peroxidation has been suggested being the destructive process in liver injury due to CCl_4 administration. This leads to liver injury and hepatocyte transport function gets disturbed resulting in leakage of plasma membrane and finally the serum enzyme levels get increased. [30]

One of the most important indicators of hepatocyte injury induced by CCl_4 administration is the release of intra cellular enzymes such as transaminases and serum alkaline phosphatases. [31] The enzymes being cytoplasmic in location are released into circulation after damage. [32] The most remarkable pathological characteristics of CCl_4 induced hepatotoxicity are fatty liver, cirrhosis and necrosis, as a result of formation of reactive intermediates. The extent of hepatic damage is assessed by the increase in the levels of serum SGOT, SGPT, SAKP, serum bilirubin and cholesterol. The rise in SGOT, SGPT, SAKP and bilirubin is due to the damage in the structural integrity of liver. It is leaked

into circulation and is associated with massive centrilobular necrosis, ballooning degeneration and cellular infiltration of liver. [33] Hence, measurement of liver enzyme has become a powerful tool in assessment of hepatotoxicity.

SGOT and SGPT are well known diagnostic indicators of liver diseases, SAKP is a membrane bound enzyme and its elevation in plasma indicates membrane disruption in the organ. [34] Measurement of liver enzyme has become a powerful tool in assessment of hepatotoxicity. [35] Both the Chr and Chl were shown to have significantly prevented CCl_4 induced elevation of liver serum enzymes indicating the hepatoprotective activity of the plant extract. In a dose dependent manner, both extracts at concentrations of 200 mg/kg were found to have anti-hepatotoxic effects. In comparison with the leaf ethanolic extract, root ethanolic extract was found to be more active. The reduction in elevated liver enzymes was comparable to that of the standard drug sylimarin. The synchronized action of antioxidant system is very critical for the detoxification of free radicals. The oxidative stress plays an important role in CCl_4 induced hepatic injury. [36-38] Free radical scavenging is a major antioxidant mechanism to prevent lipid peroxidation chain reaction. [39] Malondialdehyde (MDA) is a byproduct of polyunsaturated fatty acid oxidation and MDA level is a marker for oxidative stress and antioxidant status. [40] SOD converts highly reactive super oxides into hydrogen peroxide, whereas CAT enzyme and GSH converts H_2O_2 into water and oxygen and protects cells from reactive oxygen species. [41]

The plant extracts alleviated the oxidative stress caused by CCl_4 by increasing the activities of antioxidant enzymes. The hepatic tissue damage induced by CCl_4 caused lipid peroxidation which triggers the production of MDA. In the present study, it was shown that the acute CCl_4 treatment causes an elevation in MDA concentration which was later found to have decreased with the administration of Chr and Chl at a concentration of 200 mg/kg. The non enzymatic GSH protect the liver from oxidative stress. [42] The antioxidant enzyme SOD and CAT converts ROS into less toxic forms. The anti lipid peroxidative effects of both root and leaf ethanolic extracts were reported and it was found that both the extracts were effective in reducing MDA production in vitro in rat treated with ferric chloride-ascorbic acid, proving its anti lipid peroxidative effects. [5] The histopathological changes induced by CCl_4 treatment as evidenced by centrilobular and hepatic necrosis and its protection to normalcy by treatment with Chr was indicative of hepatoprotection of the extract. The improvement of histological scores proved the efficacy of the extract as an anti-hepatotoxic agent.

In the present study, both Chr and Chl at 200 mg/kg concentration were found to have anti-hepatotoxic effects. The ethanolic root extract has shown to be more effective in both reducing the liver serum marker enzymes as well as in rejuvenating and protecting liver cells which was confirmed by the histopathological study. These results to a great extent substantiate folkloric claims about the usefulness of *C. curviflora* root as an effective medicine to treat chronic liver diseases like jaundice.

Even though *C. curviflora* roots *have* been used in tribal medicine in treating jaundice, the anti-hepatotoxic effects of *C. curviflora* has not been reported so far. The present study revealed that the ethanolic root and leaf extracts of *C. curviflora* provides significant hepatoprotective activity. The activity is mainly due to the free radical scavenging potential of the plant. However, the mechanisms and activities of compounds of *C. curviflora* require further study.

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