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RESEARCH ARTICLE

Antiproliferative, Antioxidant and Hepatoprotective Activities of *Clematis gouriana* Roxb. Extracts

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Abstract *Clematis gouriana* Roxb ex DC. (Ranunculaceae) is a large vine capable of climbing tall trees. In the Indian system of medicine 'Ayurveda', the plant is used to eliminate malarial fever and headaches. The root and stem paste are applied externally to treat psoriasis, itches, and skin allergies. Diverse biological properties of *Clematis* species are due to many secondary metabolites found in different parts of the plant. In the present study, *C. gouriana* leaf extract showed significant antioxidant, hepatoprotective and antiproliferative properties as compared to a standard compound, silymarin. The LD_{50} values of methanolic and aqueous leaf extracts were 500 and 700 mg/kg body weight, respectively. Both extracts significantly reduced CCl_4-induced liver toxicity. The aqueous leaf extract decreased the levels of LPO and POX but increased the activities of SOD and CAT. Compared to ascorbic acid, which showed 97.28% inhibition against DPPH radical scavenging activity, the methanolic and aqueous extracts at 120 and 100 µg/ml concentrations showed 50.03 and 92.61% inhibition, respectively. Relative to the control drug doxorubicin (Adriamycin), the methanolic and aqueous extracts at any concentration (10–80 µg/ml) were unable to reduce the cytotoxicity of human colon cancer cell line HT29 or the human breast cancer cell line MCF7 when employing the sulforhodamine B assay.

Keywords Antioxidants · Antiproliferative activity · Aqueous extract · *Clematis gouriana* · Hepatoprotective activity · Methanolic extract · Ranunculaceae

Significance statement Effective antioxidant, hepatoprotective and antiproliferative properties of the methanolic and aqueous leaf extracts of the ethnomedicinally important plant, *Clematis gouriana* were recorded, fortifying its uses in Ayurveda.

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Introduction

Natural products will continue to play a key role in drug discovery and are therefore traditionally claimed as the cornerstones of drug discovery and development [1]. In fact, many drugs on the market available today have been discovered from natural sources, one important example being the analgesic activity of aspirin, which is so far the world's best known and most universally used medicinal agent. Natural products and their related drugs are used to treat 87% of all categorized human diseases through the action of various activities, including antibacterial, antipyretic, hepatoprotective, anticancer, anticoagulant, anti-inflammatory, antiparasitic, wound healing and immunosuppressant [2–5]. Plants are the richest source of novel chemical compounds. In indigenous systems of medicine, several plants are known to act as potent hepatoprotective

and antiproliferative drugs and their therapeutic properties have been evaluated using animal models by many investigators. An example in the Ranunculales, the same order of the test plant in this study, is *Berberis aristata* [6].

Clematis gouriana Roxb. ex DC. (Ranunculaceae) is a woody climber rarely found in the moist deciduous forest of Western Ghats. An ethnomedicinal survey in the Western Ghats region of Karnataka revealed that *C. gouriana* is being used by traditional practitioners to cure liver disorders, heal wounds, inflammation and as an antipyretic medicine. In the Indian system of medicine 'Ayurveda', *C. gouriana* roots are used to alleviate malarial fever and headaches while the root and stem paste of *C. gouriana* is applied externally to treat psoriasis, itches and skin allergy [7]. Traditional medicinal practitioners residing in the vicinity of Bhadra Wild Life Sanctuary, India, use the leaf and stem juices for treating infectious old wounds, psoriasis, dermatitis, blood diseases, leprosy, and liver and cardiac disorders [8]. However, the destruction of natural habitats and commercial exploitation of this species from natural locations has resulted in the dwindling of populations in the Central Western Ghats of India. In the present investigation, the methanolic and aqueous extracts of *C. gouriana* leaves were screened for their antioxidant, hepatoprotective and antiproliferative properties.

Material and Methods

Collection of Plant Material

Fresh leaves of *C. gouriana* were collected from Lakkavalli Reserve Forest Range of Western Ghats region of Karnataka, India. Plants were identified by comparing with an authenticated specimen deposited at the Kuvempu University herbarium, voucher specimen KUDB/FDD-80 [9].

Preparation of Plant Extracts

Fresh leaves of potted plants (Fig. 1) were washed with running tap water then by deionized water, and shade dried at room temperature for 15–20 days. Dried leaves were powdered mechanically, sieved (sieve No. 10/44) then subjected to Soxhlet extraction using analytical grade methanol (SD Fine Chem Ltd., Mumbai, India) as the solvent (50–80 °C) and double-distilled water (100 °C) for 72 h for a 250 g batch. The extracts were filtered and concentrated in vacuum under reduced pressure at 40 ± 5 °C using a rotary flash evaporator (Buchi, Flawil, Switzerland), until the solvent had completely evaporated and the sample was completely dry.

Fig. 1 Potted *Clematis gouriana* plant used for extracts

Drug Formulations for In Vivo Antioxidant and Hepatoprotective Activity

Suspensions of the methanolic and aqueous extracts of *C. gouriana* were prepared in 1% (w/v) gum tragacanth (Hi Media, Mumbai, India), which was used to suspend the test compound and standard drug (silymarin; (Ranbaxy Lab, Dewas, India) to obtain the dosage forms for oral administration in animal experiments.

Animals

Albino rats of either sex (Durga Experimental Animals, Shimoga, India), weighing about 150–200 g, were used for the present study. Animals were housed in polypropylene cages (six per cage), fed with a commercial diet (Hindustan Lever Ltd., Bangalore, India) and given water ad libitum throughout the experiment. The institutional ethical committee (Registration No. 144/1999/CPCSEA/SMG) provided permission to conduct the animal study. The staircase method was adopted to determine acute toxicity [10]. Healthy albino rats of either sex weighing 150–200 g were used to determine the safest dose. The vehicle used to

suspend the extracts for oral administration was 10% dimethyl sulfoxide (DMSO).

Evaluation of Hepatoprotective and In Vivo Antioxidant Activity

Albino rats were divided into 5 groups of 6 rats per group. Animals of group I (control) received the vehicle gum tragacanth (1 ml/kg/day; 1% w/v). Carbon tetrachloride (Merck, Mumbai, India) with olive oil (Sigma Aldrich, Mumbai, India) at 1:1 (v/v) was administered to all the animals of groups II to V at a dose of 0.1 ml/kg/day intraperitoneally (i.p.) at every 3 days for 14 days. Group III animals were treated with the standard drug silymarin (100 mg/kg/day, p.o.). Group IV received the methanolic extract (50 mg/kg/day, p.o.) and group V received the aqueous extract (70 mg/kg/day, p.o.). Drugs were administered concomitantly for 14 days. Animals of all 5 groups were sacrificed on the 14th day under light ether anesthesia. The blood sample of each animal was collected separately by carotid bleeding into 5 ml sterilized dry centrifuge tubes (Eppendorf, Chennai, India) and allowed to coagulate for 30 min at 37 °C. The clear serum, separated by centrifugation at 3000 rpm for 10 min, was subjected to biochemical analyses: total bilirubin (TB) [11], total protein (TP) [12], serum alanine transaminase (ALT), aspartate transaminase (AST) [13], and alkaline phosphatase (ALP) [14]. The liver homogenate was prepared with 0.15 M KCl and centrifuged [5430R centrifuge, Eppendorf, Hamburg, Germany)] at 800 rpm for 10 min. The cell-free supernatant was used in the lipid peroxidation (LOX) assay, and to estimate peroxidase (POX), catalase (CAT) and superoxide dismutase (SOD) activities.

Lipid Peroxidation

The LOX assay was carried out using the method of Buege and Aust [15]. Liver homogenate (0.5 ml) and 1 ml of 0.15 M KCl were used. Peroxidation was initiated by adding 100 µl of 0.2 mM ferric chloride. The reaction was run at 37 °C for 30 min and stopped by adding 2 ml of ice-cold mixture of 0.25 N HCl containing 15% TCA, 0.30% TBA, and 0.05% butylated hydroxyl toluene (BHT). The mixture was heated at 80 °C for 60 min and centrifuged at 3000 g after 10 min, and the absorbance of the cooled supernatant was measured at 532 nm with a Shimadzu UV-1800 (Kyoto, Japan) spectrophotometer. The results were expressed as 3,4-methylenedioxyamphetamine (MDA) (Merck) equivalents, calculated by using an extinction coefficient of 1.56–105 M^{-1} cm^{-1} mg of liver homogenate protein at 25 °C.

Estimation of Peroxidase Activity

The POX assay was carried out using the method of Nicholos [16]. To liver homogenate (0.5 ml), 1 ml of 10 mM KI solution and 1 ml of 40 mM sodium acetate solution were added. Absorbance was read at 353 nm with a Shimadzu UV-1800 spectrophotometer. Hydrogen peroxide (20 µl of 15 mM H_2O_2) was added and the change in absorbance in 5 min was recorded. Units of POX activity were expressed as the amount of enzyme required to change the optical density by 1 unit/min mg of liver homogenate protein.

Estimation of Catalase Activity

The CAT assay was carried out using the method of Aebi [17]. Liver homogenate (1 ml) from groups I, II, III and IV was added to 1.9 ml of phosphate buffer in separate test tubes (50 mM, pH 7.4). The reaction was initiated by the addition of 1 ml of 30 mM H_2O_2. The decrease in optical density due to the decomposition of H_2O_2 measured at the end of 1 min was recorded against a blank (2.9 ml of phosphate buffer + 1.0 ml of H_2O_2) at 240 nm by a Shimadzu UV-1800 spectrophotometer. Units of CAT were expressed as the amount of enzyme which decomposes 1 µM H_2O_2 min^{-1} mg of liver homogenate $protein^{-1}$ at 25 °C.

Estimation of Superoxide Dismutase Activity

The SOD assay was based on the reduction of yellow solution of nitrotetrazolium chloride (NBT) to water-insoluble purplish-blue diformazan using the method of Beauchamp and Fridovich [18]. Liver homogenate (0.5 ml) was added to 1 ml of 50 mM sodium carbonate, 0.4 ml of 24 µM NBT, and 0.2 ml of 0.1 mM ethylene diaminetetraacetic acid (EDTA). The reaction was initiated by adding 0.4 ml of 1 mM hydroxylamine hydrochloride. Zero-time absorbance was measured at 560 nm then after 5 min at 25 °C. The control was simultaneously run without liver homogenate. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50%. The specific activity was expressed in terms of units per milligram of protein.

Histopathological Studies

Liver samples were excised from the animals of each group after draining the blood and washed with normal saline. Initially, excised tissues were fixed in 10% buffered neutral formalin for 48 h. They were processed for paraffin embedding. Sections were taken at 5 µm thickness using a microtome (model MEDIMEASMRM-1120A), processed

in 10% neutral formalin solution for 24 h and dehydrated in an alcohol-xylene series and stained with Alum-haematoxylin and eosin [19]. Sections were examined microscopically with an Olympus CX41RF (Tokyo, Japan) to evaluate histopathological changes.

In Vitro Antioxidant Activity

Free-radical scavenging activity of the methanolic and aqueous extracts at different concentrations was tested in vitro. Stock solutions of the extracts were prepared by dissolving 100 mg of dried extracts in 100 ml of methanol to make a stock solution of 1 mg/ml. Aliquots from this stock solution were further diluted with methanol to obtain the final concentrations, namely 20, 40, 60, 80, 100 and 120 µg/ml.

Assay for Antiradical Activity with DPPH

Antiradical activity was measured by the decrease in absorbance at 516 nm of a methanolic solution of colored 2, 2-diphenyl-1-picrylhydrazyl (DPPH) brought about by the sample [20]. A stock solution of DPPH (1.3 mg/ml in methanol) was prepared such that 75 µl of this stock in 3 ml methanol gave an initial absorbance at 515 nm of 0.9. A decrease in absorbance in the presence of test methanol and aqueous extracts at different concentrations was noted after 15 min. EC_{50} (the concentration of the test solution resulting in a 50% decrease in the absorbance as compared to that of the blank solution) was calculated from percentage inhibition. A blank reading was obtained using methanol instead of the test sample. Ascorbic acid (99% Sigma Aldrich) was used as the standard. The percentage inhibition of free radicals was calculated in % inhibition according to the following formula:

$$\% \text{ Inhibition} = \left(\frac{(A_c - A_t)}{A_c}\right) \times 100$$

where $A_{control}$ is the absorbance of the control and A_{test} is the absorbance of the sample reaction. IC_{50} value was determined on the basis of absorbance of the control and the test sample and percentage of inhibition of different sample concentrations.

Estimation of Superoxide Anion Scavenging Activity

Superoxide anion scavenging activity of methanolic and aqueous extracts was determined by a slightly modified method. Approximately 1 ml of NBT solution containing 156 µM NBT dissolved in 1.0 ml of 100 mM phosphate buffer (pH 7.4), 1 ml NADH solution containing 468 µM NADH dissolved in 1 ml 100 mM phosphate buffer (pH 7.4), and 0.1 ml of various concentrations of test samples and the reference compound, BHT (20, 40, 60, 80, 100 and 120 µg) were mixed and the reaction was started by adding 100 µl of phenazine methosulfate solution containing 60 µM phenazine methosulfate in 100 mM phosphate buffer (pH 7.4). The reaction mixture was incubated at 25 °C for 5 min and absorbance at 560 nm was measured against control samples. A decrease in absorbance of the reaction mixture indicated an increase in superoxide anion scavenging activity. All tests were performed in triplicate. The scavenging ability of the superoxide anion radicals was calculated using the following equation [21]:

$$\% \text{ scavenging effect} = \left(\frac{(A_0 - A_1)}{A_0}\right) \times 100$$

where A_0 is the absorbance of the control (without test samples) and A_1 is the absorbance of test samples.

Evaluation of Antiproliferative Property

Human colon cancer cell line HT29 and human breast cancer cell line MCF7 (ACTREC, Mumbai, India) were grown in RPMI 1640 medium, containing 10% fetal bovine serum and 2 mM L-glutamine. For this screening, cells were inoculated into 96-well microtiter plates (Eppendorf, Chennai, India) in 100 µl of cell lines cultured at plating densities depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to the addition of plant extract. After 24 h, one 96-well plate containing 5×10^3 cells/well was fixed in situ with trichloroacetic acid (TCA) to represent a measurement of the cell population at the time of drug addition (T_z). Experimental drugs were initially solubilized in 100 mg/ml DMSO and diluted to 1 mg/ml using sterilized distilled water and stored at − 20 °C prior to use. At the time when the drug was added, an aliquot of frozen concentrate (1 mg/ml) was thawed and diluted to 100, 200, 400 and 800 µg/ml with culture medium containing the test sample. Aliquots of 10 µl of different drug dilutions were added to the appropriate microtiter wells already containing 90 µl of medium, resulting in the required final drug concentrations of 10, 20, 40 and 80 µg/ml. After adding the plant extracts, plates were incubated at 37 °C for 48 h and the assay was terminated by the addition of cold (4 °C) TCA. Cells were fixed in situ by the gentle addition of 50 µl of cold 30% (w/v) TCA (final concentration = 10% TCA) and incubated for 60 min at 4 °C. The supernatant was discarded and the plates were incubated for 20 min at room temperature. After staining, the colour stain was removed by washing five times with 1% acetic acid. All the plates were air dried. Bound stain was subsequently eluted with 10 mM Trizma® (Sigma-Aldrich) base and the absorbance was read on a microplate

plate reader (Spectro star Nano, BMG Labtech, Germany) at 540 nm with 690 nm serving as the reference wavelength. Values were recorded for GI_{50} (50% growth inhibition), TGI (total or 100% growth inhibition, which indicates the cytostatic activity the test agent), and LC_{50} (50% lethal concentration, which indicates the cytotoxic effect of the test agent).

Results and Discussion

The methanolic and aqueous extracts of *C. gouriana* leaves showed potent hepatoprotective activity as compared to the control. CCl_4-induced liver damage increased the levels of ALT, AST, ALP, TB, and TP (Table 1). The following biochemical and histological parameters were also considered to assess the normal functions, toxic destruction and pathology of hepatocytes. The biochemical estimation of blood serum of the CCl_4-treated animal group showed elevated levels of serum total bilirubin (3.22 mg/dL) indicating liver damage due to CCl_4 absorption. The standard drug, silymarin, and the CCl_4-treated group showed an almost normal level of TB, indicating the protection of hepatic cells. The animals treated with the methanolic extract showed a significant reduction in TB level (1.71 mg/dL) indicating the potency of the tested plant extract for suppressing hepatic damage caused by CCl_4. A moderate decrease in the TB was noticed in the blood samples of animals treated with the aqueous extract (1.78 mg/dL). There was a significant increase in the levels of AST, ALT and ALP. The aqueous extract was most effective in maintaining the liver function marker enzymes (AST, ALT and ALP) relative to the control. These results are shown in Table 1. After 72 h of observation, a plot of mortality values *versus* log dose showed that the LD_{50} of methanolic and aqueous extracts was 500 and 700 mg/kg body weight, respectively. About 1/10th of these doses were considered as a safe dose for oral administration in rats.

The oxidative stress marker studies revealed that chronic administration of the aqueous extract decreased the levels of LPO and POX while SOD and CAT activities increased (Table 2). The chemo-preventive effect of the aqueous extract was evident against CCl_4-induced liver cirrhosis. Treatment of rats with a single dose of CCl_4 at 1.25 ml/kg body weight significantly reduced the activities of CAT and SOD by 1.60 ± 0.15 and 0.15 ± 1.22, respectively, and increased the activity of LPO and POX by 8.50 ± 0.14 and 9.70 ± 7.07, respectively. LOX activity increased twofold more than the control after CCl_4 treatment. However, pretreatment of rats with the methanolic and aqueous extracts at 100 and 75 mg/kg restored CAT and SOD activity and inhibited LPO and POX activities. This revealed the antioxidant effect of the methanolic and aqueous extracts in protecting and maintaining the activities of these antioxidant enzymes against CCl_4 treatment (Table 2).

Histopathological studies showed that in the control, hepatocytes with normal architecture and portal triad, portal veins, and a hepatic artery and vein were observed (Fig. 2a). However, CCl_4-treated rats showed total loss of hepatic architecture, areas of hemorrhage, and necrosis (Fig. 2b). The standard drug-treated animals showed normal architecture and portal triad, portal veins, and a hepatic artery and vein (Fig. 2c). In rats pretreated with both extracts followed by exposure to CCl_4, the liver retained a normal hepatic architecture with few areas of hemorrhaging between the columns of hepatocytes, indicating the protection provided by both extracts against free radical-induced peroxidation (Fig. 2d, e). Massive generation of free radicals in the liver with CCl_4-induced damage provoked a sharp increase in lipid peroxidation. In CCl_4-treated animals, total protein level decreased due to alteration of the albumin: globulin ratio. The serum albumin level decreased slightly while globulin level showed a moderate increase. Administration of methanol and aqueous extracts to the rats elicited immediate recovery in the levels of serum protein.

Table 1 Effect of crude methanolic and aqueous extract of *Clematis gouriana* on serum biochemical parameters of CCl_4-intoxicated rats

Group (N)	Total bilirubin (mg/dl)	Total protein (g%)	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
Control (gum tragacanth, 1% w/v)	1.54 ± 0.21	8.25 ± 0.43	153.55 ± 4.04	82.11 ± 4.41	143.17 ± 2.50
Negative control (CCl_4)	3.22 ± 1.08	5.38 ± 6.45	2110.33 ± 172.75	860.30 ± 44.25	431.60 ± 10.11
CCl_4 + silymarin	1.45 ± 6.45**	6.95 ± 9.78**	210.11 ± 6.60**	93.73 ± 4.53**	225.66 ± 3.99**
CCl_4 + methanolic extract	1.71 ± 3.11**	5.79 ± 4.55**	227.62 ± 26.18**	93.32 ± 7.30**	232.79 ± 9.68
CCl_4 + aqueous extract	1.78 ± 4.27**	5.85 ± 2.04**	215.15 ± 3.99**	103.39 ± 8.42**	362.37 ± 38.59*
F-value	66.55	35.08	99.97	61.01	28.72

Each value represents mean ± S.E.M. of 4 animals. *$P < 0.05$, **$P < 0.01$ when compared to control

Table 2 In vivo antioxidant activity of methanolic and aqueous extracts of *Clematis gouriana* against CCl_4-induced oxidative stress

Treatment	CAT (IU/min/mg of liver tissue)	SOD (unit/min/mg of liver tissue)	LPO (nmol of MDA/mg of liver tissue)	POX (unit/min/mg of liver tissue)
Normal (gum tragacanth, 1% w/v)	3.82 ± 0.14	0.40 ± 0.13	3.60 ± 0.15	3.95 ± 0.11
Negative control (CCl_4)	1.60 ± 0.15	0.15 ± 1.22	8.50 ± 0.14	9.70 ± 7.07
Silymarin + CCl_4	3.85 ± 0.22**	0.40 ± 0.13**	4.47 ± 0.23*	4.80 ± 0.36*
Methanolic extract of *C. gouriana* + CCl_4	3.40 ± 0.15*	0.32 ± 6.29*	4.40 ± 0.21*	4.67 ± 0.37*
Aqueous extract + CCl_4	3.75 ± 0.23**	0.35 ± 5.00*	4.37 ± 0.19*	4.65 ± 0.43*
F-value	12.33	1.58	13.83	17.86

Each value represents mean ± S.E.M. of 4 animals. *$P < 0.05$, **$P < 0.01$ when compared to control

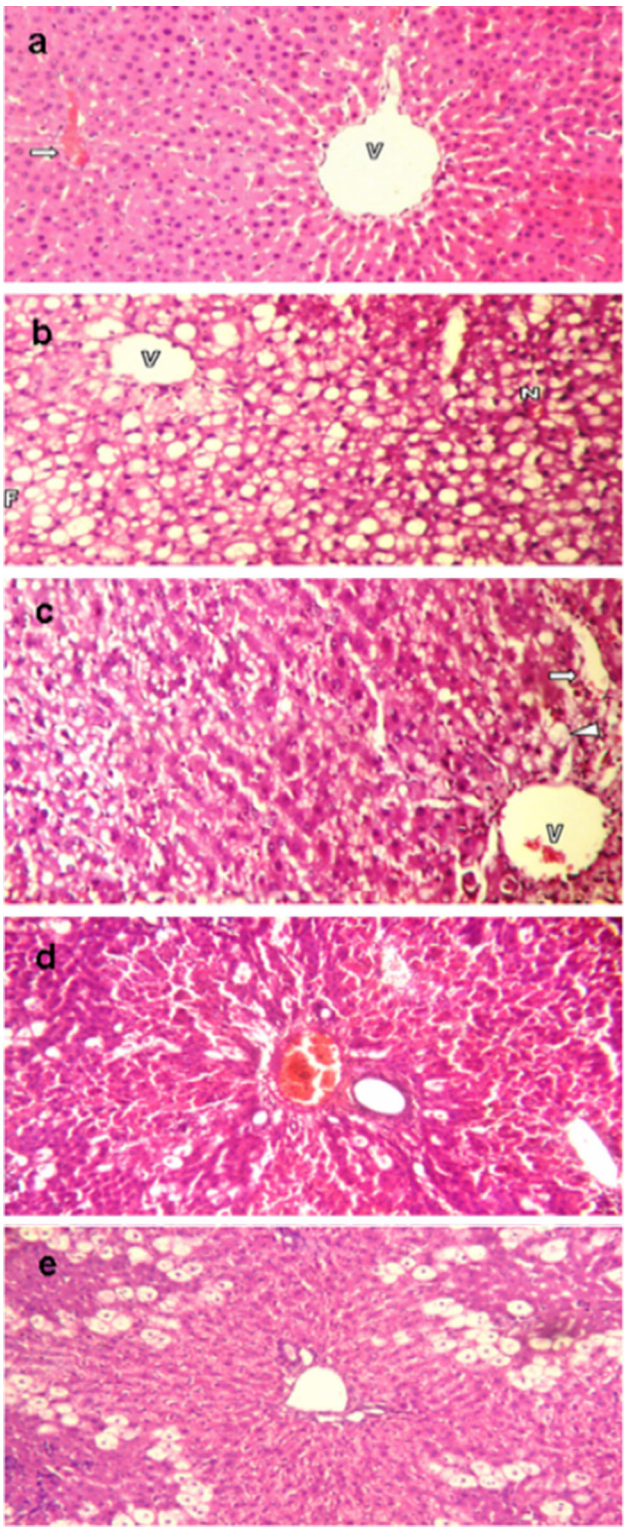
The DPPH radical scavenging activity in both extracts was expressed as a percentage of inhibition of antiradical activity. The decrease in optical absorbance at 517 nm after the addition of test compounds was monitored. The methanolic and aqueous extracts at 120 and 100 μg/ml showed 50.03 and 92.61% inhibition as compared to ascorbic acid, which showed 97.28% inhibition (Table 3). The aqueous extract at 75 μg/ml showed significant inhibition of DPPH activity and increasing antioxidant activity at different concentrations of extracts. However, it exhibited a significant dose-dependent inhibition of DPPH activity, with an IC_{50} at 100 μg.

The superoxide anion scavenging activity of methanolic and aqueous extracts at 120 and 100 μg/ml, respectively showed 49.51 and 57.66% inhibition as compared to the standard reference BHT (Table 4).

The crude methanolic and aqueous extracts of *C. gouriana* were evaluated for their in vitro cytotoxicity in an assay using human colon cancer cell line HT29 from human colon and human breast cancer cell line MCF7 from human breast by employing the sulforhodamine B (SRB) assay. The *C. gouriana* extracts were not potent cytotoxic agents according to NCI (USA) guidelines for extracts, with a $GI_{50} \leq 20$ μg/ml, which is considered to be active. The anticancer study was carried out with the assistance of the Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Mumbai. The results are depicted in Tables 5 and 6. LC_{50}, TGI and GI_{50} were calculated and compared with the positive control drug doxorubicin (adriamycin, ADR). The methanolic and aqueous extracts were not significantly active in the assay system used. The LC_{50}, TGI and GI_{50} values for both cell lines were > 80 μg/ml whereas ADR showed an LC_{50} value > 80 μg/ml but TGI and GI_{50} values of 49.8 μg/ml and < 10 μg/ml, respectively for the HT29 cell line; and 61.1 μg/ml, 26.4 μg/ml and < 10 μg/ml, respectively for the MCF7 cell line. The biochemical observations, supported by the histopathological examination, revealed that both extracts had potent properties of antioxidants, hepatoprotective and anticancer activities.

Discussion

The liver is an important internal organ in the body as it maintains a homeostatic balance of the physiological activities. The unique ability of the liver to regenerate is essential because the liver actively participates in detoxification and is exposed to various toxins. Chronic liver injury occurs as a result of a prolonged process with persistent hepatic injury due to various causes. CCl_4, when injected, is readily converted into a trichloromethyl radical ($CCl_3^{\cdot}$) that reacts with oxygen molecules to form a trichloromethyl peroxy radical ($CCl_3OO^{\cdot}$) [22]. These free radicals thus formed cause hepatocellular damage by reacting with cellular molecules through the initiation of lipid peroxidation. The toxic effect of CCl_4 is due to free radical generation ($CCl_3^{\cdot}$), which leads to the formation of lipid peroxides, which in turn leads to the production of malondialdehyde (MDA) that causes damage to cellular membranes. This lipid peroxidative degradation of biomembranes is one of the principal causes of CCl_4 hepatotoxicity [23] by encouraging the auto-oxidation of fatty acids present in the cytoplasmic membrane phospholipids and causes functional and morphological changes in the cell membrane, thus altering the permeability of liver cell membranes [24]. This is evidenced by the elevation of serum marker enzymes namely AST, ALP and ALT in response to the administration of CCl_4 in experimental rats. The rise in serum levels of AST and ALT has been attributed to the damaged structural integrity of the liver because these are cytoplasmic in location and are released into circulation after cellular damage [25, 26]. This suggests disturbance in the transport function of hepatocytes resulting in leakage of enzymes from cells due to altered permeability of membranes. In the present investigation,



◀**Fig. 2** Sections of liver tissue of rats. **a** Section of the liver tissue of the CCl_4-treated rats showing total loss of hepatic architecture and areas of hemorrhaging. **b** Section of the liver tissue of rats pretreated with standard drug followed by exposure to CCl_4; the liver shows normal hepatic architecture with few areas of hemorrhaging between the columns of hepatocytes. **c** Section of the liver tissue of rats pretreated with silymarin followed by exposure to CCl_4, the liver shows normal hepatic architecture. **d** Section of the liver tissue of rats pretreated with the methanolic extract of *C. gouriana* leaves followed by exposure to CCl_4, the liver shows normal hepatic architecture with a few areas of hemorrhaging between the columns of hepatocytes. **e** Section of the liver tissue of rats pretreated with an aqueous extract of *C. gouriana* leaves by exposure to CCl_4; the liver shows normal hepatic architecture. A, artery; D, hepatic duct; F, fatty vacuoles; N, necrosis; V, vein. **a**–**c** H&E ×100. **d**, **e** H&E ×40

the levels of serum marker enzymes increased significantly in those animals treated with CCl_4. A concomitant administration of the methanolic extract with CCl_4 showed a significant reduction in the serum enzyme levels.

The aqueous extract was highly effective in reducing the toxic effect of CCl_4 by controlling the levels of serum marker enzymes AST, ALP and ALT. This effect was comparable to that of the standard drug silymarin. ALP is a membrane-bound glycoprotein enzyme present in high concentrations in the sinusoids and the endothelium of the central and periportal veins [27]. ALP is involved in the transport of metabolites across cell membranes, protein synthesis, and synthesis of certain enzymes, secretory activities and glycogen metabolism. Thus, the rise in serum ALP activity in rats induced with CCl_4 damage may be due to a disturbance in the secretory activity or in the transport of metabolites, or may be due to altered synthesis of certain enzymes as in other hepatotoxic conditions. The level of serum ALP increased significantly in animals treated with CCl_4. In contrast, a concomitant administration of methanol and aqueous extract with CCl_4 showed a significant reduction in the elevated level of serum ALP. This effect was comparable to that of the standard drug silymarin. Measurement of serum bilirubin has been reported to be a sensitive indicator of liver injury [25, 28]. The substantially elevated serum enzymatic levels of AST, ALP, ALT, TB and TP in CCl_4-treated animals were restored to almost normal levels by both extracts. Bilirubin is produced by the normal breakdown of red blood cells. Normally bilirubin passes through the liver and is excreted as bile through the intestines. Jaundice occurs when bilirubin builds up faster than the liver can break it down and pass it from the body. The causes of hepatic jaundice include acute hepatitis, hepatotoxicity, biliary cirrhosis and alcoholic liver disease, whereby cell necrosis reduces the liver's ability to metabolize bilirubin and the excess bilirubin is recycled back to blood. The increase in the levels of serum bilirubin reflects

Table 3 DPPH free radical scavenging activity of methanolic and aqueous extracts of *Clematis gouriana*

Compounds	Percentage of scavenging activity					
	20 µg	40 µg	60 µg	80 µg	100 µg	120 µg
Ascorbic acid	82.11 ± 0.79*	96.37 ± 0.45*	97.21 ± 0.02*	97.17 ± 0.03*	97.28 ± 0.37*	97.74 ± 0.24**
Methanolic extract	1.84 ± 0.24	9.06 ± 0.57	16.36 ± 0.29	25.72 ± 0.25	39.63 ± 0.19	50.03 ± 0.58*
Aqueous extract	64.59 ± 0.07*	72.29 ± 0.02*	79.31 ± 0.25*	89.32 ± 0.02*	92.61 ± 0.40**	95.83 ± 0.64**
F-value	9599.0	9953.0	7046.0	7228.0	5019.0	5361.0

Each value represents mean ± S.E.M. of 3 observations.*$P < 0.01$, **$P < 0.001$ when compared to control

Table 4 Superoxide anion scavenging activity of methanolic and aqueous extracts of *Clematis gouriana*

Compounds	Percentage of scavenging activity					
	20 µg	40 µg	60 µg	80 µg	100 µg	120 µg
BHT	58.49 ± 0.2*	67.07 ± 0.3**	72.62 ± 0.4**	80.60 ± 0.1**	81.98 ± 0.1**	83.37 ± 0.2**
Methanolic extract	3.64 ± 0.68	29.85 ± 0.7	33.18 ± 0.45	42.27 ± 0.1*	45.55 ± 0.3*	49.51 ± 0.3*
Aqueous extract	32.74 ± 2.2*	47.32 ± 0.5*	50.65 ± 0.5*	55.36 ± 0.2*	57.66 ± 0.2*	60.08 ± 0.6**
F-value	1223.0	1705.0	2500.0	5866.0	6395.0	1225.0

Each value represents mean ± S.E.M. of 6 observations. *$P < 0.01$, **$P < 0.001$ when compared to control

Table 5 In vitro anticancer data of crude methanolic and aqueous extracts of *Clematis gouriana* on human colon cancer cell line HT29 (% growth compared to the control)

Treatment	% Growth			
	10 µg/ml	20 µg/ml	40 µg/ml	80 µg/ml
Methanolic extract	111.1 ± 0.12*	112.6 ± 0.2*	113.4 ± 0.17	109.1 ± 0.24**
Aqueous extract	106.3 ± 0.07*	104.5 ± 0.02*	107.4 ± 0.29*	110.6 ± 1.12
Adriamycin (ADR)	3.7 ± 0.13	0.5 ± 0.24*	3.8 ± 0.62*	− 16.8 ± 0.37*

Each value represents mean ± S.E.M. of 6 observations. *$P < 0.01$, **$P < 0.001$ when compared to control

Table 6 In vitro anticancer data of crude methanolic and aqueous extracts of *Clematis gouriana* on human breast cancer cell line MCF7 (% growth compared to the control)

Treatment	% Growth			
	10 µg/ml	20 µg/ml	40 µg/ml	80 µg/ml
Methanolic extract	95.3 ± 0.62*	87.8 ± 0.09*	98.7 ± 1.13	104.7 ± 0.01**
Aqueous extract	90.9 ± 0.17	89.4 ± 0.01**	93.3 ± 0.5*	102.3 ± 0.25*
Adriamycin (ADR)	− 14.6 ± 0.23*	− 22.8 ± 0.12*	− 37.9 ± 0.1**	− 58.0 ± 0.64*

Each value represents mean ± S.E.M. of 6 observations. *$P < 0.01$, **$P < 0.001$ when compared to control

the depth of jaundice. Therefore, in the present investigation, the CCl_4-intoxicated rats displayed hyperbilirubinemia, a common sign of hepatic jaundice. A decrease in serum bilirubin after treatment with the methanolic extract in damaged livers indicates the effectiveness of the extract in the normal functional status of the liver.

Stimulation of protein synthesis is a contributory hepatoprotective mechanism that accelerates the regeneration and production of liver cells [29, 30]. This may reveal the immediate recovery and regeneration of polysomes and endoplasmic reticulum membranes, thus emphasizing the crucial importance of antioxidants in the protection of the

liver. Assay systems for in vitro cytotoxicity study used in an earlier study [30] were human colon cancer cell line HT29 from the human colon and human breast cancer cell line MCF7 from human breast by employing the sulforhodamine B (SRB) assay.

Conclusion

The present investigation strongly supports the ethnomedical use of *C. gouriana.* The methanolic and aqueous extracts of *C. gouriana* afforded significant protection against CCl_4-induced hepatotoxicity by decreasing the hepatotoxic serum marker enzymes levels towards normalcy and acting as a free radical scavenger by intercepting those radicals evolved during ethanol metabolism by the microsomal enzymes and hindering the interaction of oxygen-related free radicals with polyunsaturated fatty acids. Both *C. gouriana* extracts did not significantly abolish lipid peroxidation, nor were they effective cytotoxic agents. Thus, *C. gouriana* extracts may be a promising drug for acute cases of liver disorders as the extracts are non-cytotoxic in nature.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflicts of interest to publish this manuscript.

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