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The potential mitigating activity of *Cassia absus* seed extract on carbon tetrachloride-induced liver injury

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ABSTRACT

Introduction: Carbon tetrachloride (CCl_4) is a chemical that can induce injury in the liver. The aim was to evaluate any potential hepatoprotective potential of a seed extract from *Cassia absus* against CCl_4-induced liver toxicity in Wister rats.

Methods: To this end, an aqueous-methanolic extract of *C. absus* seeds was prepared by maceration. In vitro testing of the extract included phytochemical screening and high-performance liquid chromatography (HPLC) analysis to evaluate the phenolic compound constituents. An in vivo study involved a single exposure to CCl_4 either alone or in combination with the hepatoprotective agent, silymarin, or *C. absus* seed extract administered orally over 28 days. Serum biochemical markers of liver cell injury were measured and post-mortem liver tissues were examined histopathologically using eosin-hematoxylin staining and microscopy.

Results: The HPLC analysis specifically identified the presence of gallic acid, vanillic acid, catechin, and p-coumaric acid. In addition, no changes were observed in animal body and liver weights during the treatment protocol. However, both the plant seed extract and silymarin reversed the CCl_4 induced elevated serum concentrations of aspartate aminotransferase (AST), total bilirubin, alkaline phosphatase (ALP), and alanine transaminase ($P<0.05$) in addition to the histopathological injury. The plant extract also had a dose-related hepatotoxic mitigating effect, and the findings were analogous to those with the hepatoprotective standard comparator, silymarin.

Conclusion: These study outcomes substantiate a protective effect of *C. absus* seed extract against CCl_4-induced hepatotoxicity in the animal model.

Implication for health policy/practice/research/medical education:
Aqueous methanolic extract of *Cassia absus* possesses significant hepatoprotective activity and might be used as adjunctive therapy to prevent liver injury caused by chemicals, especially CCl_4.

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Introduction

The liver has a pivotal role in protein synthesis, chemical detoxification, bile secretion, and bilirubin excretion, in addition to the metabolism of hormones, fats, and carbohydrates. This key organ is susceptible to hepatic injury which may arise from a variety of causes including exposure to toxic chemicals, for example, carbon tetrachloride (CCl_4) or thioacetamide, bacterial and viral infections, misuse of antibiotics, heavy alcohol consumption or drugs. Exposure to such harmful entities has the potential to induce pathologies ranging from cirrhosis, hepatitis, hepatic encephalopathy, liver failure, and even tumors like hepatoma (1). Typically, hepatotoxic agents instigate liver toxicity by promoting lipid peroxidation, oxidative stress in hepatocytes, and serum levels of biochemical markers of hepatocyte injury (2).

Carbon tetrachloride is a recognized chemical sometimes employed to incite hepatotoxicity in animals

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as an experimental model. It incites hepatotoxicity after a short exposure, and predominant symptoms include convulsions, weakness, vertigo, and coma (3). An underlying mechanism of hepatic injury entails oxidative stress in hepatocytes and CCl_4 toxicity leads to activation of the cytochrome P-450 system yielding the potentially reactive trichloromethyl peroxyl radical (CCl_3*) (4). This radical attacks lipid membranes to initiate a chain reaction causing lipid peroxidation and eventually, hepatocellular damage along with a potential risk of cancer (5).

With the widespread use of medicinal plants as complementary treatments for hepatic damage, phytochemical-containing herbal medications have garnered increased interest over recent years. In this context, ayurvedic ethnomedical records exist for the plant *Cassia absus*, which belongs to the Fabaceae family. In traditional medicine, it is used to treat a variety of ailments including bronchitis, asthma, headaches, hemorrhoids, conjunctivitis, renal and hepatic illnesses, constipation, tumors, and venereal ulcers (6). Studies have also revealed that the seeds of *C. absus* possess antibacterial (7), antidiabetic (8), antifertility (9), antihypertensive (10), antiglycation, and alpha-amylase inhibitory actions as well as antioxidant properties (11). Given its antioxidant activity, and after a review of the scientific literature regarding this medicinal plant, it was established that no study to date, has been conducted regarding any hepatoprotective potential. In this context, oral therapy with the accepted hepatoprotective agent N-acetylcysteine (NAC) is poorly tolerated due to vomiting (12), and the natural compound, silymarin, does not affect all-cause mortality in cirrhosis patients but it does facilitate the prevention of liver-related mortality (13,14). Consequently, this study evaluated any in vitro or in vivo propensity of *C. absus* seed extract against CCl_4-induced liver injury in rats as a conceivable lead to unveiling a potentially useful oral hepatoprotective agent.

Materials and Methods

Preparation of *Cassia absus* seed extract

Cassia absus seeds were purchased from the Bahawalpur district of Pakistan. Following identification by the Botany Department, The Islamia University, Bahawalpur, the seed samples were preserved in the herbarium of the Department of Pharmacology, and a voucher number (CA-SD-08-21-201) was issued for future reference. The plant seed extract was prepared by maceration and extraction (70/30 (v/v) methanol to water) and then dried under reduced pressure in a rotary evaporator. The thick viscous paste obtained was stored at -20 °C in a sealed flask for subsequent usage.

Chemicals

Chemicals were all of analytical grade, including CCl_4 (Sigma Aldrich, Germany), silymarin (Abbott Laboratories), sodium chloride (Pakistan Chemical), methanol (Sigma Aldrich, Germany), and chloroform, (Sigma Aldrich, Germany). The following chemicals were obtained from the sources in brackets: xylazine (Prix Pharmaceuticals, Pakistan), and ketamine (Global Pharmaceuticals). The test kits for aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin and alkaline phosphatase (ALP) were obtained from Humans Diagnostics.

In vitro studies

Phytochemical evaluation

High-performance liquid chromatography (HPLC) analysis was performed to establish any flavonoid and phenolic content in the *C. absus* extract. For this purpose, a 50 mg sample of the extract was dissolved in distilled water (5.0 mL) plus methanol (12 mL) and then incubated (5 minutes). Next, 6.0 mL distilled water was added and the resultant mixture was incubated (5 minutes). Subsequently, HCl (10 mL, 15 M) was added and the mixture was incubated for 2 hours (90 °C) in order to disrupt glycoside linkages. The solution was then filtered with a 0.2 µm syringe filter and the samples were injected onto an HPLC shim-pack CLC-ODS (C-18) column. The mobile phase consisted of acetonitrile: dichloromethane: methanol (60:20:20) and the flow rate was adjusted to 1.0 mL/min. Phenolic and flavonoid compounds were detected with a UV-Vis detector set at 280 nm (15).

In vivo studies

Experimental animals

Normal healthy Wistar albino rats of either sex, weighing 190 ± 20 g were purchased from the vivarium of the Faculty of Pharmacy, The Islamia University of Bahawalpur, Pakistan. The rats were provided with a normal rodent diet with ad libitum access to water, and after acclimatization, they were randomly assigned from different home cages to five groups (n = 6) by an independent researcher who was "blind" to the treatments. In the laboratory, conditions were maintained at a temperature of 25.0 ± 3.0 °C (humidity > 65%), on an alternating 12 h/12 h light/dark cycle.

Carbon tetrachloride-induced hepatotoxicity protocol

To determine any hepatoprotective activity, animal groupings were as follows:

- Group A: the control group, received distilled water vehicle (3.0 ml/kg PO) once.
- Group B: the hepatotoxic group, received a single dose of CCl_4 (3.0 ml/kg PO).
- Group C: the standard comparator group (16), received a single dose of CCl_4 (3.0 ml/kg PO) and silymarin (25 mg/kg PO), which was administered after two days of CCl_4 treatment up to the 28th day of the protocol.
- Group D: the first treatment group that initially received CCl_4 (3.0 mL/kg PO) and after two days,

the animals were administered aqueous methanolic extract of C. *absus* (300 mg/kg PO),

- Group E: the second treatment group firstly received CCl_4 (3.0 ml/kg PO) and after two days treated with aqueous methanolic extract of C. *absus* (600 mg/kg PO) and continued till the end of the protocol (17).

Assessment of liver biochemical and histopathological parameters

The weight of all experimental animals was measured on protocol days 0, 14, and 28. On days 0 and 14, all animals were anesthetized with ketamine (500 mg/10 mL) plus xylazine (23.32 mg/mL) at a 2.0 mL/kg dose, and post-mortem blood samples were collected by retro-orbital puncture in EDTA tubes. Following centrifugation at 3000 rpm for 15 minutes, the resultant serum was stored in a refrigerator at -4 °C. On protocol day 28, the animals were fasted overnight, weighed, and euthanized using the ketamine and xylazine anesthesia mixture. Serum was collected for hematological analysis. Post-mortem, livers were immediately dissected, washed with normal saline, and stored in 10% formalin for histopathological analysis.

Histopathological analysis

Liver tissue samples were subjected to standard histopathological techniques using graded methanol, fixation with xylene, and followed by embedding in paraffin wax. Staining of slides was performed with eosin and hematoxylin stain, and images were viewed on an optical microscope at 10-40× resolution (18).

Statistical analysis

Data were presented as mean ± SEM (n=6). The Shapiro-Wilk test was applied to confirm the normal distribution of the data. Then, statistical significance between different experimental groups was evaluated by two-way ANOVA with post hoc Bonferroni's test with the aid of GraphPad Prism (version 8.0). Values of $P<0.05$ were considered statistically significant.

Results

The *C. absus* plant extract was brown and yielded 12% from 1.0 kg of seeds.

High-performance liquid chromatography

HPLC divulged the presence of the phenolic compounds gallic acid, P-coumaric acid, catechin, and vanillic acid in the *C. abs*us seed extract (Figure 1). Gallic acid was detected at the highest concentration, while vanillic acid was identified as the lowest concentration in the plant seed extract (Table 1).

Effect of *Cassia absus* seed methanolic extract treatment on rat liver and body weights following exposure to CCl_4

Animal body weights were measured throughout the study (protocol days 0, 14, and 28), and post-mortem liver weights were recorded on day 28, following exposure to CCl_4. No significant difference was noticed by any treatments during the course of the protocol (Table 2).

Assessment of liver biochemical and histopathological parameters

Effects of Cassia absus on CCl_4-induced serum biochemical measures

Treatment of rats with CCl_4, by the end of the protocol, elevated serum levels of AST (+219.8%), alanine transaminase (ALT; +440.1%), ALP (+146.6 %), and total bilirubin (+444.4 %), whereas total protein in serum was reduced (-23.07%) (Figure 2). Both silymarin and *C. absus* seed extract at the 3 higher dose (600 mg/kg) restored these CCl_4 modified biochemical measures to levels which were not significantly different from controls ($P>0.05$). In the case of *C. absus*, (600 mg/kg), there was also a reversal

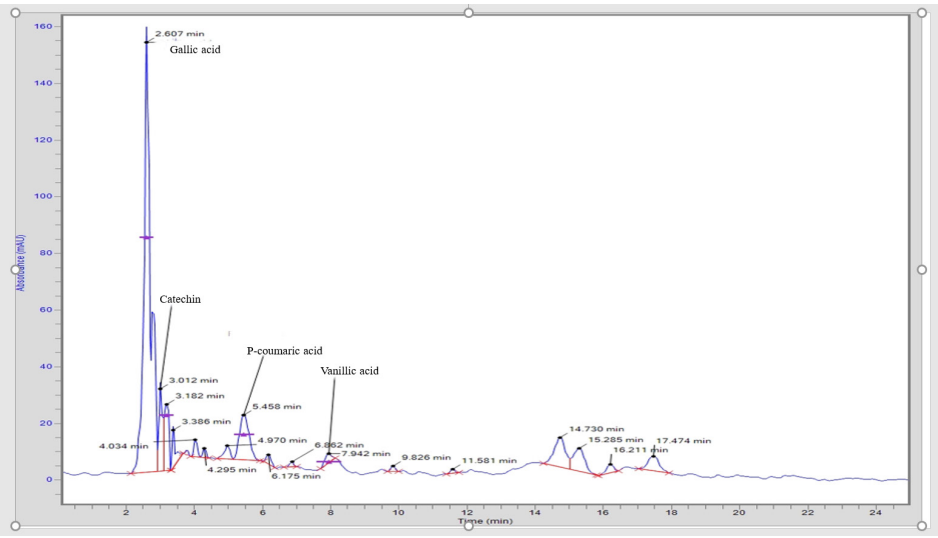

Figure 1. Chromatogram showing different phenolic compounds in methanolic extract of *Cassia absus* seeds.

Table 1. Concentration of various phenolic compounds in *Cassia absus* seed methanolic plant extract

No	Component	Retention time (min)	Quantity (mg/kg)
1.	Gallic acid	2.607	166.9
2.	Catechin	3.012	83.90
3.	P-coumaric acid	5.458	92.89
4.	Vanillic acid	7.942	1.877

of reduced total protein to a level that was not significantly different from control (P >0.05) (Figure 2).

Effects of Cassia absus on CCl_4-induced hepatic histopathology

Liver tissue samples from groups were prepared for histopathological evaluation to establish any possible hepatoprotective activity of *C. absus*. Samples from the control group exhibited normal histology (Figure 3A). No signs of inflammation, degeneration, or necrosis were evident; however, the CCl_4-treated animal group tissue samples disclosed atrophy in hepatocytes, and there was localized aggregation of lymphocytes (arrow), indicating an immune response shown in Figure 3B. Samples from the CCl_4 plus silymarin-treated group (Figure 3C) displayed minimal infiltration of inflammatory cells within the sinusoidal space. Additionally, the *C. absus* extract treated groups showed binucleated hepatocytes (arrow) that were indicative of active mitosis and regeneration of liver cells at a dose of 300 mg/kg (arrows in Figure 3D); however, at 600 mg/kg, the hepatocytes displayed normal cytoplasm and normal histopathological architecture (Figure 3E) suggestive of a hepatoprotective proclivity.

Discussion

The initial study aimed to identify phytochemical components in the aqueous methanolic seed extract of *C. absus* and to evaluate any possible hepatoprotective activity against CCl_4 hepatotoxicity in the experimental animals. The extraction method using maceration in an aqueous methanolic solution (30/70% v/v) has been previously proven to be an effective way of extracting organic constituents (19). A subsequent qualitative phytochemical analysis of the plant seed extract has previously demonstrated that it contained phenols, alkaloids, tannins, glycosides, flavonoids, saponins, and quinones (20). These findings accord with an earlier report that a hydroalcoholic *C. absus* seed extract had similar constituents with a high phenolic content (21). Our quantitative HPLC analysis of the seed extract disclosed that catechin, gallic acid, vanillic acid, and p-coumaric acid were constituents. Among these isolated phenolic compounds, gallic acid was present at the highest concentration, and it has previously been demonstrated as a potent hepatoprotective agent (22) via the inhibition of free radical formation, provoking lipid peroxidation and suppression of elevated liver enzymes (23). Moreover, the antioxidant p-coumaric acid produces hepatoprotection through the inhibition of mitogen-activated protein kinases (MAPKs) and apoptosis signaling by augmenting nuclear factor erythroid 2 related factor (Nrf2) signaling (24). Equally, vanillic acid has been shown to decrease the accumulation of collagen and the level of hydroxyproline during hepatic fibrosis induced by CCl_4 (25,26). It also inhibits the activation of hepatic stellate cells without modifying hepatocyte viability (27,28). What is more, catechins have a noteworthy role in regulating glucose metabolism and genes implicated in lipid synthesis, along with a beneficial impact on oxidative stress-related pathways capable of activating pro-inflammatory effects conducive to liver damage (29).

CCl_4 was used to induce liver damage in vivo, through its ability to catabolize free radical-induced lipid peroxidation. This causes membrane damage, swelling, and necrosis of hepatocytes, which initiates the release of cytosolic enzymes (total bilirubin, AST, ALP, and ALT) into the bloodstream (30). Our findings demonstrated that a single dose of CCl_4 also elevated the concentrations of total bilirubin, ALT, AST, and ALP, but decreased the level of total protein. Treatment with the standard agent silymarin and two doses of *C. absus* seed extract restored liver enzyme levels, and this outcome was more pronounced with the higher dose of the seed extract. These findings were also supported by the histopathological studies in liver tissues whereby animals treated with CCl_4

Table 2. The effects of *Cassia absus* seed methanolic extract on rat liver and body weights treated with carbon tetrachloride (CCl_4)

Treatment groups	Animal weight (g)			Liver weight (g)
	Protocol day 0	Protocol day 14	Protocol day 28	Protocol day 28
Control group	195.17 ± 13.13	206.83 ±12.37	211.67 ± 12.51	6.27 ± 0.28
CCl_4	183.33 ± 3.63	186.33 ± 6.42	189.00 ± 8.35	6.75 ± 0.29
Silymarin	179.67 ± 10.33	185.50 ± 11.33	197.17 ± 10.87	5.58 ± 0.36
C. absus extract (300 mg/kg)	188.50 ± 9.56	204.00 ± 10.38	217.00 ± 10.52	6.42 ± 0.42
C. absus extract (600 mg/kg)	182.33 ± 10.21	188.67 ± 10.72	199.67 ± 12.89	5.63 ± 0.39

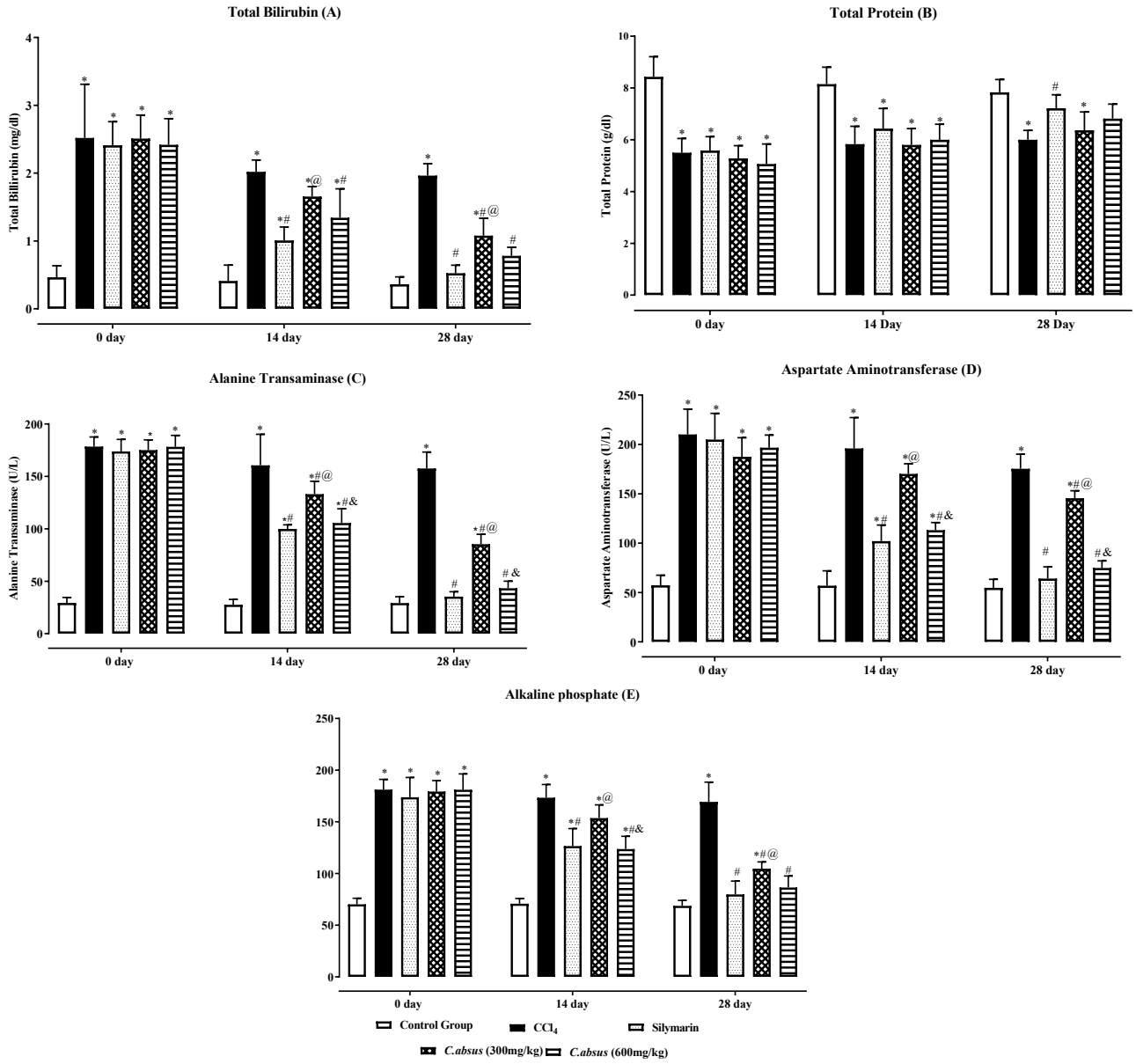

Figure 2. Effect of *Cassia absus* on serum biochemical parameters in carbon tetrachloride (CCl_4)-induced hepatotoxicity in rats (mean ± SD, n=6). (**A**) Total bilirubin, (**B**) total protein, (**C**) alkaline transaminase, (**D**) aspartate transaminase, and (**E**) alkaline phosphate serum levels of control, CCl_4 treated, and CCl_4 co-treatment groups of rats [CCl_4 + silymarin, CCl_4 + *C. absus* (300 mg/kg), CCl_4 + *C. absus* (600 mg/kg)]. *P < 0.05 versus control; #P < 0.05 versus CCl_4; @P <0.05 versus CCL_4 + silymarin; &P < 0.05 versus CCl_4 + *C. absus* (300 mg/kg).

displayed adverse tissue changes, while treatment with either *C. absus* extract or silymarin tended to normalize and maintain tissue integrity.

In our in vivo experiments, the standard comparator agent, silymarin, has previously been shown to reduce liver-related deaths in clinical trials (30). Such an effect has been ascribed to antioxidant-free radical scavenging, in addition to modifying enzyme systems connected with hepatocellular injury (16). Consequently, silymarin is an effective hepatoprotective agent and the current findings indicate that *C. absus* seed extract may well have a similar ability to induce a comparable intensity of hepatoprotection.

Conclusion

Arising from the current study, it was concluded that the aqueous methanolic extract of *C. absus* seeds was comparable to the standard reference agent, silymarin, in its intensity of ability to protect rats from acute liver damage caused by CCl_4. Our identification of the antioxidant phenolic constituents, gallic acid, catechin, p-coumaric acid, and vanillic acid in the plant seed extract, will predictably contribute to its activity. Accordingly, we are currently conducting further research with the isolated compounds to better understand the precise mechanism underlying *C. absus* hepatoprotective activity.

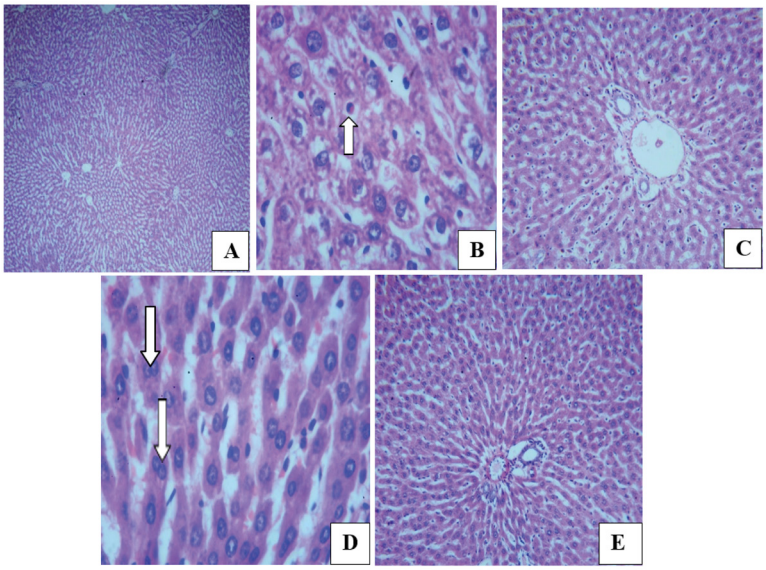

Figure 2. Effect of *Cassia absus* on serum biochemical parameters in carbon tetrachloride (CCl_4)-induced hepatotoxicity in rats (mean ± SD, n=6). (**A**) Total bilirubin, (**B**) total protein, (**C**) alkaline transaminase, (**D**) aspartate transaminase, and (**E**) alkaline phosphate serum levels of control, CCl_4 treated, and CCl_4 co-treatment groups of rats [CCl_4 + silymarin, CCl_4 + *C. absus* (300 mg/kg), CCl_4 + *C. absus* (600 mg/kg)]. *P < 0.05 versus control; #P < 0.05 versus CCl_4; @P <0.05 versus CCL_4 + silymarin; &P < 0.05 versus CCl_4 + *C. absus* (300 mg/kg).

Limitations of the study

Since this is an initial study designed to establish if there is any hepatoprotective propensity of *C. absus* seed extract, no specific analysis of the constituents against liver damage has currently been performed. However, the investigation has now positively confirmed that the plant extract is hepatoprotective, so a logical step might involve an evaluation of a combination of the constituents ostensibly in the proportions occurring in the extract.

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Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author's contribution

Conceptualization: Fiaz-ud-Din Ahmad.
Data curation: Ifrah Hussain.
Formal analysis: Robert D. E. Sewell, Shahzad Khan, Muhammad Shahzad Chohan.
Funding acquisition: Shahzad Khan, Muhammad Shahzad Chohan.
Investigation: Ifrah Hussain.
Project administration: Fiaz-ud-Din Ahmad.
Software: Ammara Asif.
Supervision: Fiaz-ud-Din Ahmad.
Writing-original draft: Ammara Asif, Robert D. E. Sewell.
Writing-review & editing: Robert D. E. Sewell.

Ethical considerations

The study protocol was approved by the Pharmacy Animal Ethics Committee, Faculty of Pharmacy, The Islamia University of Bahawalpur, (certificate #PAEC/21/56).

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