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Study the hepatoprotective effect of the methanolic extract of *Plumbago auriculata* Lam. against CCl_4-induced hepatocyte damage in mice

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Abstract

The liver is an important organ in the body; its diseases are considered the major causes of morbidity and mortality around the world. Hepatotoxic chemicals cause damage to liver cells. Medicinal plants have a powerful hepatoprotective effect. This study has been aimed to assess the potential hepatoprotective effect of the methanolic extract of *Plumbago auriculata* Lam. against carbon tetrachloride (CCl_4) - induced hepatocyte damage in albino mice. The methanolic extract of the plant was subjected to preliminary phytochemical analysis to determine the presence of secondary metabolites according to standard protocols. The acute toxicity study was carried out to measure the LD_{50}. Swiss albino mice were divided into 4 groups treated intraperitoneally (IP) once daily for 7 days; the first group was a negative control, while the second group, considered as a CCl_4 model, received a single dose of carbon tetrachloride during the last day. The third group received 500 mg/kg of body weight of methanolic extract of *P. auriculata,* and the last group received 100 mg/kg of body weight of standard silymarin, after 2 h of treatment, on the last day, all animals (except negative control group) have received CCl_4 at a dose of 1 mL/kg of body weight. Biochemical analysis of collected blood and histopathological examination were performed. Results revealed that the preliminary phytochemical screening of the methanolic extract of the plant confirmed the presence of many biologically active secondary metabolites like flavonoids, steroids, terpenoids, saponins, glycosides and phenols and LD_{50} of the extract was 2.5 g/kg. The CCl_4-intoxicated mice showed an increase in biochemical enzyme levels (ALT and AST) compared with the negative control group. The extract of 500 mg/kg revealed a significant reduction ($p \leq 0.05$) in enzyme activities. The histopathological analysis of plant extract and silymarin-treated groups revealed a decrease in the pathological features compared with the CCl_4-intoxicated group. It was concluded that the extract of *P. auriculata* has a significant effect on hepatoprotection against CCl_4-induced hepatocyte damage and this may be because of the combined effects of the bioactive compositions of plant extract.

Keywords

Plumbago auriculata ; antioxidant; silymarin; intraperitoneal

Introduction

The liver is an important organ in the body due to its multi-biological functions in the metabolism of carbohydrates, proteins and lipids. It participates

in the role of regulating, maintaining and performing body homeostasis (1). Liver is a vital tool in detoxifying and excreting exogenous and endogenous constituents (2). The main reason for morbidity and mortality around the world is liver diseases. There are many factors that cause liver diseases directly or indirectly, such as obesity, viruses, diabetes and exposure to chemicals or drugs. In addition, untreated liver diseases and many autoimmune disorders can cause liver cancer and malignancy and eventually death (3).

The hepatotoxic materials can cause the destruction of liver tissues through induction and elevation of oxidative stress, tissue lipid peroxidation and the levels of several biochemical parameters like cholesterol, bilirubin, triglycerides, transaminases and alkaline phosphatase (4). One of the most important models of xenobiotics, which causes induction of free radical-mediated hepatotoxicity in animals, is carbon tetrachloride (CCl_4). It would be converted by cytochrome-P450 into free radicals of trichloromethyl (CCl_3) and proxy trichloromethyl ($OOCCl_3$), which can initiate the peroxidation of lipid and liver damage (5, 6).

Medicinal plants have been screened for antioxidant compounds, that exert a powerful hepatoprotective effect by enhancing antioxidant status (7). Thus, natural antioxidants possess considerable attention due to their ability to protect the liver from oxidative destruction and they can avoid liver disease (8).

Many medicinal plants have a wide range of therapeutic uses. In recent years, they have been used for their lesser toxicity, few side effects and great effect on dynamic healing when compared with their synthetic counterparts (9). *P. auriculata* Lam. is a medicinal herb belonging to the Plumbaginaceae family, and it is a permanent shrub present in South Africa. It was being inserted in tropical and subtropical places in America, Asia and Europe. This species is very well acclimatized and normally found in farms in Brazil, which flower all over the year (10). Some species of *Plumbago* were used in traditional remedies to treat warts, wounds, headache and fractures, giving as an anticancer, antimicrobial, anti-inflammatory and hepatoprotective activities (11). *P. auriculata* contains many bioactive constituents including steroids, terpenoids, flavonoids and naphthoquinones with the last 2 constituents considered as chemo-systemic parameters of the plant family (12). This study aims to assess the potential hepatoprotective effect of the alcoholic extract of *P. auriculata* against carbon tetrachloride-induced hepatocyte damage in albino mice.

Materials and Methods

Chemicals and reagents

Normal saline and vegetable oil were obtained from the local market. Absolute methanol, CCl_4, ether, 10 % formaldehyde, liquid paraffin, paraffin wax, hematoxylin, eosin, xylene, the standard drug of silymarin and assay kits for liver analysis (Sigma-Aldrich, Germany).

Collection and authentication of plant materials

The mixture of *Plumbago auriculata* aerial parts was collected from a local farm in Baghdad city in July 2022. The plant was classified by the taxonomist at the Biology Department, College of Science, Baghdad University (Specimen number was 1305). Parts of the plant have been cleaned, left in the shade to dry at room temperature and grinded in a mechanical mortar to a coarse powder.

Plant extract preparation

The powdered plant (100 g) has been extracted using the Soxhlet extraction technique with 85 % methanol as a solvent to obtain the crude extract. When the extraction was completed, the solvent became colorless in the siphon tube. The extract was filtered and subjected to drying using a rotary evaporator and the extractive value was calculated.

Preliminary phytochemical screening

Preliminary phytochemical investigation was performed on the methanolic extract of *P. auriculata* to determine types of secondary metabolites present in this plant using standard procedures for alkaloids, flavonoids, phenols, steroids, terpenes, cardiac glycosides and saponins (13-15).

Experiment

Healthy male Swiss albino mice used in this experiment were weighed between 25–30 g and their ages were 3 months, inbred in the house of the animal at the pharmacy college of Al-Nahrain University. All mice were retained in cages of 6 mice each at 25 °C and 12 h : 12 h light and dark schedule and they were given water and commercial pellets freely and adapted to the environment of the laboratory for 1 week before the start of the experiment.

Study of acute toxicity

The acute toxicity of methanolic extract of *P. auriculata* was evaluated according to the World Health Organization (WHO) guideline (16). Experimental mice have been divided into four treated groups of 5 mice each. Before the experiment, mice were fasted for 12 h except from water. The plant extract was prepared freshly (dissolved in distilled water) at 5, 2.5, 1.25 and 0.625 g/kg body weight (17, 18). Then, the plant extract was injected once daily by intraperitoneal (IP), depending on the body weight. Treated mice were continuously monitored for any sign or symptom of toxicity like diarrhea, weight loss, hair loss, ataxia, tachycardia, lacrimation, water intake and death for 30 min and then intermittently followed for 4 to 24 h after administration of the treatment. The mice were further observed for up to 14 days following the treatment for any behavioral change and mortality (19).

Assessment of hepatoprotective effect

The hepatoprotective effect of the alcoholic extract of *P. auriculata* was assessed by a CCl_4-induced hepatotoxic model in albino mice. All mice were fasted overnight before the experiment except for free water. Animals were divided into 4 groups of 5 each and the extract was injected intraperitoneally once daily for a week.

Group-I: used as negative control (N) received distilled water only (300 µL).

Group-II: used as CCl_4 model group (M) received a single dose of carbon tetrachloride on the last day.

Group-III: used as a treated group (T) received the crude extract (500 mg/kg) body weight.

Group-IV: used as positive control received silymarin (S) (100 mg/kg) body weight (20).

Then, after 2 h of the last administration of the treatment, on the last day, all animals from each group (except the negative control group) were given CCl_4 at a dose of 1 mL/kg of body weight (1:3 diluted in vegetable oil) by intraperitoneal injection (21), while the negative control group was merely given the same amount of vegetable oil (22). All experimental animals were fasted for 12 h (22). The blood was collected from the jugular vein for biochemical analysis (17, 23). All mice were euthanized by cervical dislocation to get histopathological analyses of the liver after conserving the liver in 10 % formaldehyde (18, 22).

Blood biochemical analysis

The total samples of blood were centrifuged at 4000 rpm for 10 min at 3 °C to analyze the Aspartate aminotransferase (AST) level as well as Alanine aminotransferase (ALT) level (24).

Histopathological study

The tissue of the liver was fixed with 10 % formaldehyde and performed normally for implanting in paraffin wax. Liver sections of 5-6 µm thick have been prepared for staining with eosin and hematoxylin (E and H) dye to observe the histopathological changes of the liver using microscope at 100x and 400x scales (25, 26).

Statistical analysis

The results of biochemical analysis have been introduced as mean ± standard deviation. A significant difference in the four groups has been measured by one-way analysis of variance, then post hoc t-test as provided by SPSS version 24 and considered value of $p \leq 0.05$ as a significant difference (27).

Results

Plant extract preparation

Powdered plant was extracted with 85 % methanol using the Soxhlet extraction technique. The % yield of the crude extract was 6.25 %.

Preliminary phytochemical screening

The results of the phytochemical analysis are given in Table 1.

Acute toxicity study

As a preliminary step to assess the toxic effect of *Plumbago auriculata* extract, the acute toxicity was carried out to evaluate the lethal dose of 50 % (LD_{50}) and safety of the plant extract. The number of surviving mice in each group was recorded. After management of the crude extract intraperitoneally at different concentrations, the % of death of mice was 100 % at dose 5 g/kg and 80 % at dose 2.5 g/kg, while the percentage was 0 % at dose 1.25 and 0.625 g/kg. Table 2 shows the signs of toxicity that were observed. Therefore, the dose-response curve was drawn from the counting of both the % of death and dose injected into mice to determine LD_{50}%, as shown in Fig. 1, where the lethal dose was 2.5 g/kg.

Hepatoprotective study

Blood biochemical analysis

The data in Fig. 2 showed an effect in the levels of ALT and AST as demonstrated in Table 3, where (N) was the nega-

Table 1. Preliminary phytochemical analysis of *Plumbago auriculata* plant.

No.	Chemical group	Test	Result	Appearance
1.	Alkaloids	Dragendorff's test	Negative	Absence of reddish- orange precipitate
2.	Flavonoids	Sodium hydroxide test	Positive	Yellow- orange color
3.	Phenols	Ferric chloride test	Positive	Dark greenish-blue color
4.	Steroids	Liebermann-burchard test	Positive	Dark green color
5.	Terpenes	Salkowski test	Positive	A reddish brown coloration at the interface
6.	Cardiac glycosides	Keller-kiliani test	Positive	Green-blue color
7.	Saponins	Froth test	Positive	Froth that was persistent more than 10 minutes

Table 2. Signs observed after administration of methanolic extract in mice.

No.	Dose gm/kg	Signs of toxicity	Number of mice dead
1.	5	Ataxia, calmness, loss of the ability of movement, tachycardia, shallow breathing, diarrhea, lacrimation, decreased in bodyweight and water intake	All
2.	2.5	Sweating, ataxia, calmness, hair loss, hypoactivity, decreased in bodyweight, sunken eye, decreased in water intake.	2
3.	1.25	Neither sign of toxicity nor death	0
4.	0.625	Neither sign of toxicity nor death	0

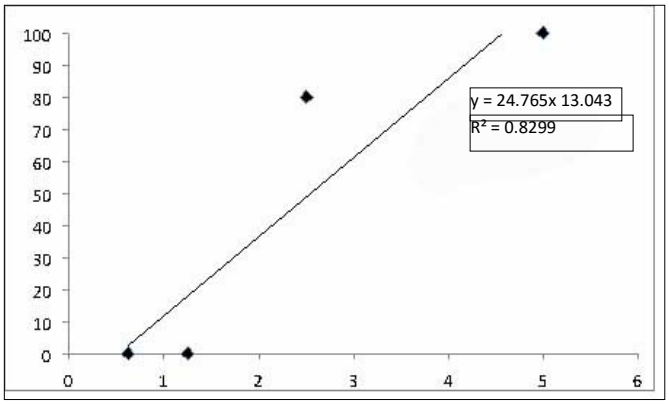

Fig. 1. The dose-response curve of methanolic extract in mice where Y-axis represents the % of dead mice while X-axis represents the dose in g/kg.

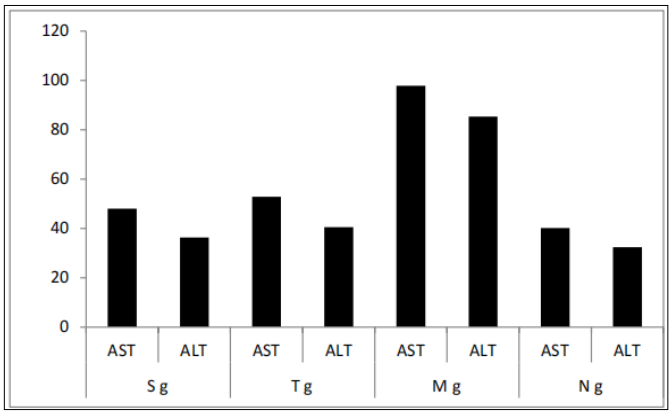

Fig. 2. Effect of studied plant extract on Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) against CCl_4-induced hepatotoxicity in mice. Where X-axis represents dose of S, T, M and N groups in mg/kg and Y-axis represents concentrations of ALT and AST in U/mL ± SD.

Table 3. Effects of the methanolic extract of *Plumbago auriculata* on biochemical enzymes in CCl_4 induced hepatotoxicity in mice.

No	Treatment	Dose mg/kg	ALT U/mL	AST U/mL
1.	Negative control group	-	32.33 ± 3.2	40.61 ± 7.13
2.	CCl_4 model group	1 mL/kg	85.33 ± 8.11	97.8 ± 6.55
3.	Plant extract group	500 mg/kg	40.5 ±2.25	52.38 ± 6. 91
4.	Silymarin group	100 mg/kg	36.33 ±2.58	48 ± 4.37

tive control group, (M) was the CCl_4 model group, (T) was the treated group of crude extract and (S) was a positive control group of silymarin. Data of results were introduced as mean ± standard deviation (SD).

Results have shown a significant increase in the enzyme levels in the serum of mice treated with the hepatotoxic agent of (the CCl_4 group (M)) compared to the negative control group (N) ($p \leq 0.05$). Animals which have been treated with an intraperitoneal concentration (500 mg/kg) of methanolic extract of the plant (T group) revealed a significant improvement in AST and ALT levels, while their levels were decreased significantly in comparison with CCl_4-intoxicated mice (M group) ($p \leq 0.05$). The effect of 500 mg/kg of the methanolic extract on the biochemical parameters was comparable to those of silymarin (S group), which has non-significant differences in enzyme activities compared to the S group ($p \geq 0.05$).

Histopathological study

Histopathological analysis of the liver of the negative control group appeared regular arrangement of hepatic cords, normal central vein, and sinusoids as shown in Fig. 3. Focal necrosis with aggregation of mononuclear cells (MNCs) and marked steatosis within hepatocytes were observed in CCl_4-intoxicated group, Fig. 4. While there were signs of protection revealed in the sections of liver of mice that were given a dose of 500 mg/kg of plant extract as appeared by the reduction/absence of steatosis and necrosis, affected to a significantly less degree than CCl_4-intoxicated group which revealed marked disarrangement of hepatic cords with multiple hemorrhagic foci and marked focal aggregation of MNCs as shown in Fig. 5. The liver sections of mice received 100 mg/kg of silymarin showed normal histological structures, Fig. 6.

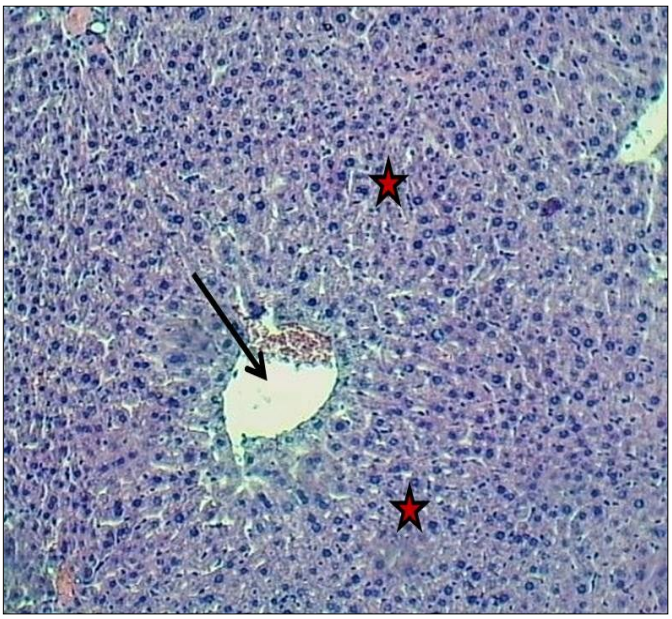

Fig. 3. Section of liver (negative control) shows: normal arrangement of hepatic cords (Asterisks), normal central vein (Arrows) and sinusoids. H & E stain.100x.

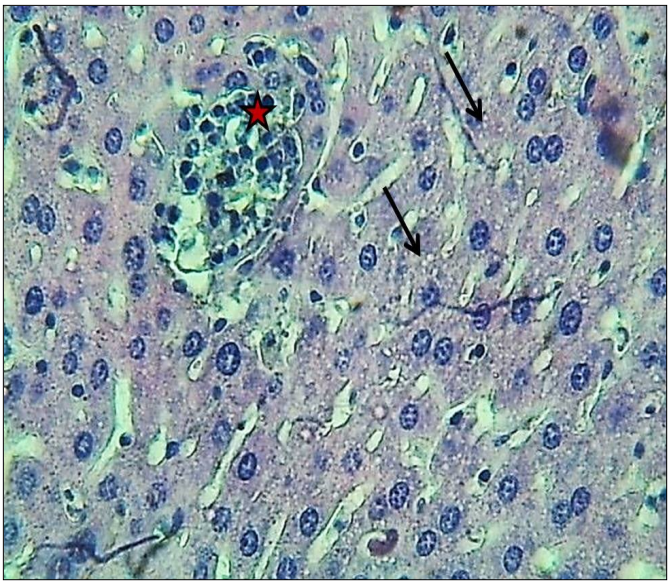

Fig. 4. Section of liver (CCl_4) shows: focal necrosis with aggregation of MNCs & marked steatosis within hepatocytes (Arrows). H & E stain.400x.

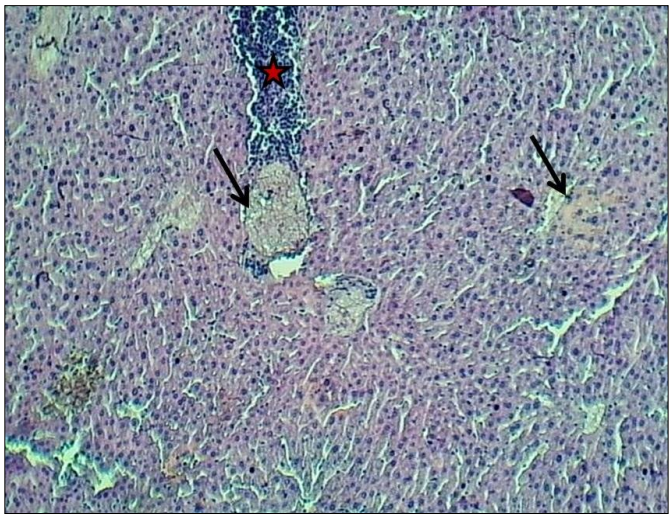

Fig. 5. Section of liver (plant group) shows: marked disarrangement of hepatic cords with multiple hemorrhagic foci (Arrows) and marked focal aggregation of MNCs (Asterisk). H & E stain.100x.

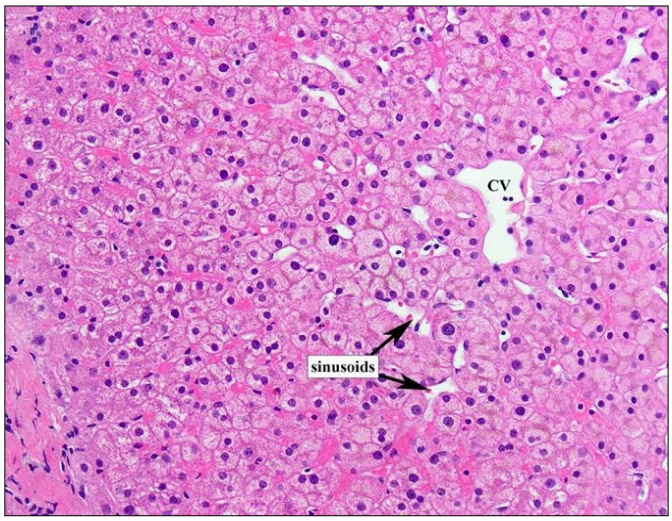

Fig. 6. Section of liver (silymarin) shows: normal appearance of hepatocytes, central vein and sinusoids. 100x.

Discussion

The results of the preliminary phytochemical analysis of the methanolic extract of *Plumbago auriculata* revealed the presence of bioactive constituents such as flavonoids, phenols, steroids, saponins, terpenes and cardiac glycosides, which exhibit a wide range of biological properties, including antioxidant, antimicrobial, anti-inflammatory and antidiabetic activity.

The initial step in screening natural products for pharmacological activity is an estimation of the toxic features of plant extract to determine the LD_{50} and the safety of the extract. Due to the classification of toxicity, materials that have a median lethal dose in the range between 1–5 g/kg have been estimated to be low toxic, while the materials that have higher values of more than 5 g/kg have been estimated as non-toxic materials (28). Results of this study have indicated that the LD_{50} was 2.5 g/kg when administered intraperitoneally and that was considered safe in the methanolic extract of the plant because it showed no signs of toxicity or death of mice in high dose during 14 days, as this was concluded from the acute toxicity study conducted on albino mice. These results have been attributed to the bioactive contents that appeared in the plant extract, such as steroids, flavonoids, naphthoquinones and terpenoids, in safe concentrations (12).

The study aimed to assess the hepatoprotective effect of the *P. auriculata* against carbon tetrachloride (CCl_4) intoxicated albino mice. As the extract has a high margin of safety, the initial dose to be used for hepatoprotection was 500 mg/kg.

Results showed that induction of liver toxicity by CCl_4 causes a significant elevation in enzyme activity in serum compared to the negative control group; this result was consistent with other studies (29, 30). The metabolism of CCl_4 by the liver to highly reactive metabolites leads to lipid peroxidation of the hepatic cells either directly or indirectly (31). Consistent lipid peroxidation can cause leakage of the cytosolic liver enzymes into circulation. Increased enzyme activities are closely associated with liver degradation, demonstrated by cellular permeation, enlargement, degeneration and massive centrilobular necrosis of the liver (32). Alanine aminotransferase has been considered a specific indicator of liver destruction caused by a viral infection, toxic drugs and alcohol (33).

According to the data, levels of these biochemical parameters (AST, ALT) of the treated group with 500 mg/kg of methanolic extract were lower than the CCl_4 model group level and appeared to have an approximately similar effect as silymarin standard group; this indicates the dose of the extract (500 mg/kg) may have a significant protection effect due to the existence of bioactive constituents which has been investigated by chemical tests such as steroids, terpenoids, phenolic, saponins, tannins, flavonoids and naphthoquinones having free radical scavenging activity, antioxidant and prevention of lipid peroxidation (34-37). These biochemical results of the extract were further confirmed by the results of histopathological analysis, as the severity of injury, necrosis and steatosis caused by CCl_4 were significantly decreased in mice treated with the extract.

The bioactive compounds that appeared to have the antioxidant and hepatoprotective effects of the plant extract were not identified; it was suggested that the bioactive constituents of the plant might work individually or together, producing the hepatoprotective that was observed. Perhaps the naphthoquinones and flavonoids present in this extract are responsible for hepatoprotection through scavenging of free radical ability, lipid peroxidation inhibition, and preventing the damage of cells according to previous studies (36, 38-40).

Conclusion

From this study, we concluded that the methanolic extract of *Plumbago auriculata* is very rich in secondary metabolites such as flavonoids, phenols, steroids, saponins, terpenes and cardiac glycosides, which exhibit various therapeutic effects. Also, results of acute toxicity indicated that the alcoholic extract of *P. auriculata* has a very high margin of safety and its LD_{50} was 2.5 g/kg. Therefore, treatment of

mice with 500 mg/kg body weight of alcoholic extract appeared to have a significant hepatoprotective effect, as this was shown by decreasing the raised levels of biochemical enzymes (AST and ALT) compared with the CCl_4-intoxicated group and further confirmed by histopathological analysis. The combined effect of bioactive compounds, including natural antioxidants present in the plant extract, could be due to the hepatoprotective activity observed. More research is recommended to determine the active constituents responsible for this hepatoprotective effect.

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Authors' contributions

MNA carried out the study. AHK designed the study and performed the statistical analysis. The authors performed the critical revision. Therefore, the authors read and agreed on the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors declared that they have no conflict of interest.

Ethical issues: The present study was conducted with the scientific and ethical committees of the faculty of Pharmacy at Baghdad University.

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