

Original Research Article

Protective effect of leaf extract of *Ficus carica* L. against carbon tetrachloride-induced hepatic toxicity in mice and HepG2 cell line

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Abstract

Purpose: To determine the *in vivo* and *in vitro* hepatoprotective effects of *Ficus carica*.

Methods: The methanol leaf extract of *Ficus carica* L was further fractionated into *n*-hexane, ethyl acetate and aqueous fractions. For *in vivo* study, male albino mice were divided into twelve groups. Hepatotoxicity was induced in the mice using carbon tetrachloride (CCl₄). The extract of *F. carica* and its fractions were administered at doses of 200 and 400 mg/kg. Silymarin was used as standard hepatoprotective drug. The protective effects of the extract and fractions were determined via assay of biochemical parameters and antioxidant enzymes in the liver. The histopathology of the liver was also studied. Moreover, the *in vitro* hepatoprotective effect of the extract and fractions against CCl₄-induced damage was determined in HepG2 cell line.

Results: There were significant increases in the serum levels of liver biomarkers in CCl₄-treated group, whereas treatments with plant extract and fractions significantly reduced the levels of these parameters ($p < 0.05$). In addition, results from histopathology revealed evidence of protective effect of *Ficus carica* through reversal of CCl₄-induced decreases in the activities of liver antioxidant enzymes.

Conclusion: These results indicate that methanol leaf extract of *Ficus carica* L. and its fractions exert significant and dose-dependent hepatoprotective effects *in vivo* and *in vitro*.

Keywords: *Ficus carica*, Hepatoprotection, Carbon tetrachloride, Liver biomarkers

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INTRODUCTION

Liver diseases are serious health problems. Indeed, approximately 2 % of total deaths are due to liver disorders. The liver is a vital organ which performs many functions such as storage, metabolism, secretion and detoxification. It

maintains physiological homeostasis. The liver is the largest organ in humans, and it is susceptible to chemical-induced toxicity. Hepatic damage is initiated by over-production of reactive oxygen species (ROS) [1]. Reactive oxygen species (ROS) interact with, and modify biomolecules such as proteins, lipids and DNA, resulting in various degenerative diseases. The liver

contains a natural defense system of antioxidant enzymes comprising peroxidases, superoxide dismutase (SOD), catalase (CAT) and reductases, all of which combat ROS so as to overcome oxidative stress [2]. Carbon tetrachloride (CCl₄) is used to induce hepatotoxicity in animal models. In the liver, cytochrome p450 converts CCl₄ to trichloromethyl free radical (·CCl₃) which subsequently binds to membrane proteins and lipids, resulting in lipid peroxidation and loss of hepatocyte membrane integrity [3].

Liver diseases are mostly not completely curable with allopathic medicines, although this issue has remained controversial. Thus, current research is focused on hepatoprotective compounds obtained from plants due to the fact that these compounds have no adverse side effects. For thousands of years, several populations have relied on medicinal plants rather than synthetic drugs for their primary health care needs [4].

Ficus carica is a deciduous tree belonging to the family *Moraceae*. It grows well in tropical and subtropical regions. The plant has been used for centuries to cure different diseases. Traditionally the leaves and fruit latex of *Ficus carica* are used for treating jaundice, gout, anemia and anti-inflammatory. Studies have revealed the presence of various bioactive compounds such as phenols, flavonoids, triterpenoids, cardiac glycosides and alkaloids in the leaves, fruit, stem and latex of *Ficus carica* plant [5].

The purpose of the present study was to determine the hepatoprotective effects of methanol leaf extract of *Ficus carica* and its derived fractions against CCl₄-induced toxicity. Furthermore, the *in vitro* protective effects of the extract and its fractions were determined in HepG2 cell line.

EXPERIMENTAL

Plant material

Ficus carica leaves were collected from Islamabad, Pakistan in the month of July 2016. Authentication of sample was done by Dr Rehmatullah Qureshi, Associate Professor in Botany Department, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan. A voucher specimen of the plant was deposited in the herbarium of the University (voucher no Bot 2459).

Extraction and fractionation of plant material

After cleaning and shade-drying, the leaves were ground into fine powder using an electrical

grinder. The preparation of the extract was done using a simple maceration process. A portion of the powdered leaves was soaked in methanol for 72 h at room temperature, after which the mixture was filtered through Whatmann no 1 filter paper. Then, the solvent was evaporated under reduced pressure in a rotatory evaporator (Heidolph, Germany). The methanolic extract was mixed with water and subjected to partitioning in a separating funnel using solvents of increasing polarity (n-hexane, ethyl acetate and water). Following solvent evaporation from the extract and its fractions, the dried samples were kept 4 °C prior to use.

Animals and experimental design

Male albino mice were obtained from National Institute of Health, Islamabad. The animals were housed in stainless steel cages, with free access to water and laboratory diet. The study received approval from the ethical committee of PMAS-AAUR (approval no. PMAS_AAUR/BCH/326), and the experimental procedure used for the animals were carried out in line according to National Institutes of Health guidelines. The mice were divided into 12 groups, with 5 mice in each group. The study was performed for two weeks. Group I (normal healthy group) received normal saline only. Group II (vehicle control mice) were treated with olive oil only. Group III (toxic control mice) received CCl₄ intraperitoneally at a dose of 0.2 mL/kg. Mice in group IV were treated with the standard drug silymarin and CCl₄ twice a week. Groups V and VI mice were administered methanol extract at doses of 200 and 400 mg/kg, respectively, post-orally. Mice in groups VII and VIII were served with n-hexane fraction at doses of 200 and 400 mg/kg, respectively, in addition to CCl₄, twice a week. Group IX and group X were treated with ethyl acetate fraction at doses of 200 and 400 mg/kg, respectively, while mice in group XI and group XII received the aqueous fraction at doses of 200 and 400 mg/kg, respectively.

Biochemical analysis

Blood samples were collected from all animals using direct cardiac puncture. Serum samples were obtained by centrifugation of the blood samples at 3000 rpm for 10 min. The method described by Qureshi *et al* [6] was used for estimation of liver biomarkers i.e. alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin. The assays were done using commercially available kits in line with the kit manufacturers' protocols. Serum total protein was determined with Lowry's method [7].

Determination of activities of liver antioxidant enzymes

Liver tissue homogenate was prepared by grinding 100 mg of fresh liver tissue with 1 mL of potassium phosphate buffer (100 mM, pH 7.4). The homogenate was centrifuged at 12000 g for 30 min at 4 °C, and the supernatant was used for assay the activities of antioxidant enzymes [8]. Catalase (CAT) activity was determined using method of Chance [9], while the activity of SOD was determined using the method of Kakkar [10]. Glutathione peroxidase (GPx) activity was assayed using the method of Lawrence and Burk [11].

Histopathological studies

Liver samples dissected out from mice of all groups were fixed in 10 % formalin. The fixed tissues were processed by dehydration in increasing concentrations of ethanol, followed by embedding in paraffin. Then, 5- μ m tissue slices were sectioned, put on slides and stained with haematoxylin and eosin (H & E). The tissue slides were observed under a light microscope (Am Scope, USA) and photographed.

In vitro hepatoprotective effect of extract and fractions against CCl₄-induced damage

In vitro hepatoprotective screening was based on protection of HepG2 cells [12]. Toxicity was induced using CCl₄, and the *in vitro* hepatoprotective effect was determined using MTT assay based on mitochondrial viability. The HepG2 cell line was obtained from Institute of Biomedical and Genetic Engineering, KRL Hospital Islamabad. The cells were cultured in 96-well plates at density of 1.0 x 10⁵ cells/well in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) overnight at 37 °C in a humidified atmosphere of 5 % CO₂. A partial monolayer was formed after 24 h. The culture medium (supernatant) was removed, and the monolayer was washed. Then, the cells were exposed to CCl₄ and different concentrations of the plant extract fractions (0.1, 1 and 10 μ g/mL). After 24 h, the medium was discarded and each well was rinsed with phosphate buffered saline (PBS). Then, 0.05 % 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and incubated for 2 h. Thereafter, the MTT was aspirated off, and the resultant blue formazan crystals were dissolved in DMSO. The absorbance of the formazan solution in each well was measured at 540 nm using a microplate reader (BioRad).

Statistical analysis

Values are expressed as mean \pm standard deviation (SD). The effects of the different treatments were compared using one-way analysis of variance (ANOVA), followed by Dunnett's post-test. Computer software GraphPad prism 5.0 was used. Statistical significance was assumed at $p < 0.05$.

RESULTS

Effect of *F. carica* on liver biomarkers

As shown in Table 1, treatment with CCl₄ led to significant increases in the serum levels of liver biomarker enzymes i.e. ALT, AST and ALP, as well as total bilirubin, while the level of serum total protein was reduced, relative to the normal control mice ($p < 0.05$). However, interestingly, treatment with plant extract and fractions reversed the increases in the levels of these enzymes. The ethyl acetate fraction produced the most significant effect ($p < 0.05$). Treatment with ethyl acetate fraction at a higher dose (400 mg/kg) led to significant decreases ($p < 0.05$) in the serum levels of liver marker enzymes to levels almost similar to those in the silymarin-treated group. The least hepatoprotective potential was exhibited by the n-hexane fraction.

Effect of *F. carica* on liver enzymes

As shown in Table 2, CCl₄ reduced the activities of the liver enzymes CAT, SOD and GPx, when compared with normal control group. However, treatment with ethyl acetate fraction at doses of 200 and 400 mg/kg resulted in enhancement of the activities of these enzymes. Moreover, the methanol extract and aqueous fraction reduced the activities of the liver enzymes. The effects of the extract and fractions were dose-dependent. Maximum potential was produced at the higher dose of 400 mg/kg. The n-hexane fraction produced low oxidative potential. In addition, the *Ficus carica* extract increased serum protein levels, thereby reversing the CCl₄-induced reduction in protein levels.

Histopathological features

Liver sections from mice in the healthy group had normal hepatocyte structure, with normal sinusoidal spaces and central vein (Figure 1). However, CCl₄ administration resulted in complete loss of liver architecture, necrosis, dilation of central vein and congested sinusoidal spaces.

Table 1: Effect of *Ficus carica* L. extract and its fractions on serum biochemical parameters

Group	ALT (U/L)	AST (U/L)	ALP (U/L)	Total bilirubin (g/dL)
Normal control	34.5±3.46	32.4±2.10	63.2 ± 3.62	0.20 ± 0.27
Vehicle control	35.2±1.53	35.2± 2.81	65.6 ± 2.40	0.29 ± 1.81
Toxic control	198±3.19**	136.7±2.94**	329 ± 3.18**	2.86 ± 0.45**
Silymarin	40.5±2.14 ^{##}	48.6±3.73 ^{##}	79.2 ± 2.36 ^{##}	0.30 ± 0.97 ^{##}
ME 200 mg/kg	85.4 ± 2.96	90.2 ± 3.56	104 ± 2.45	0.56 ± 2.90
400 mg/kg	64.3 ± 3.28	61 ± 2.29	84 ± 3.19	0.34 ± 1.21 ^{##}
NHF 200 mg/kg	133.4 ± 3.91	95.9 ± 2.46	176.4± 2.57	0.48 ± 0.11
400 mg/kg	125.6 ± 3.20	84.6± 3.04	184.9 ± 2.74	0.52 ± 0.08
EAF 200 mg/kg	50.2 ± 3.15	59.0± 5.49	83.6±1.86	0.44 ± 0.26
400 mg/kg	44.9 ± 1.81 ^{##}	51 ± 3.53 ^{##}	76.2 ± 2.48 ^{##}	0.76 ± 0.11
AQF 200 mg/kg	85 ± 3.33	74.4 ± 2.61 ^{##}	126.6 ± 1.99	0.80 ± 0.71
400 mg/kg	65.6± 3.78	66.2 ± 2.86	173.2 ± 1.92	0.80 ± 0.10

Values are expressed as mean ± S.D (n =5). ** $P < 0.05$, compared to normal control group; ^{##} $p < 0.05$, compared to CCl₄ (toxic control). (ALT = alanine transaminase, AST = aspartate aminotransferase, ALP = alkaline phosphatase)

Table 2: Effect of *Ficus carica* L. extract and its fractions on liver enzymes and total protein

Group	CAT (U/mg protein)	SOD (U/mg protein)	GPx (mol/min/mg protein)	Total protein in serum (g/dL)
Normal control	7.64 ± 2.18	11.8±2.56	39.8 ± 2.94	5.90 ± 2.13
Vehicle control	5.90 ± 3.24	9.94 ± 2.49	29.3 ± 3.36	5.56 ± 1.59
Toxic control	2.20 ± 2.96*	5.40 ± 3.17*	24.2 ± 3.31*	3.38 ± 2.45*
Silymarin	6.20 ± 2.16 [#]	9.10 ± 2.16 [#]	34.6 ± 1.78 [#]	5.16 ± 1.69 [#]
ME 200 mg/kg	4.60 ± 2.97	8.14 ± 2.90	32.9 ± 2.17	5.18 ± 2.38
400 mg/kg	4.91 ± 1.16	8.66 ± 1.67	33.5 ± 2.96	5.51 ± 1.96
NHF 200 mg/kg	3.78 ± 1.18	7.72 ± 2.81	25.4± 1.89 [†]	4.00 ± 1.90
400 mg/kg	3.38 ± 3.67	7.82 ± 3.42	30.4 ± 2.67	4.34 ± 1.41
EAF 200 mg/kg	5.26 ± 2.78	8.69 ± 2.48	37 ± 1.18	5.18 ± 2.76 [#]
400 mg/kg	5.72 ± 2.3 [#]	9.00 ± 3.78 [#]	35.5 ± 1.54 [#]	5.27 ± 2.90 [#]
AQF 200 mg/kg	4.30 ± 1.98	8.00 ± 2.45	27.4 ± 2.28	4.44 ± 1.56
400 mg/kg	4.80 ± 3.78	8.20 ± 1.89	32.5 ± 2.76	4.58 ± 2.55

Values are expressed as mean ± S.D. * $P < 0.05$, compared to normal group; [#] $p < 0.05$, compared to CCl₄ toxic control. (CAT = catalase, SOD = superoxide dismutase, GPx = glutathione peroxidase)

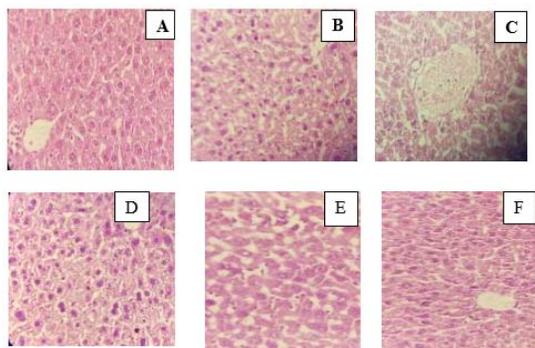


Figure 1: Histopathology of liver sections of mice. **A:** Liver section from normal control, **B:** liver section from mice treated with olive oil (olive oil control), **C:** liver section from mice given CCl₄ only, **D:** liver section from mice treated with silymarin, **E:** liver section from mice that received methanolic extract (200 mg/kg) + CCl₄, and **F:** liver section from mice given methanolic extract (400 mg/kg) + CCl₄.

These CCl₄-induced lesions were reversed by treatment with methanol leaf extract of *Ficus carica* and its fractions, as shown in Figure 2. At a dose of 400 mg/kg, the protective effect of the

ethyl acetate fraction on hepatocytes was similar to that produced by the hepatoprotective agent silymarin.

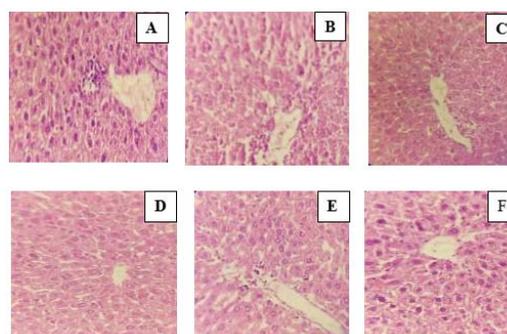


Figure 2: Histopathology of liver sections of mice. **A:** Liver section from mice given n-hexane fraction (200 mg/kg) + CCl₄, **B:** liver section from mice given n-hexane fraction (400 mg/kg) + CCl₄, **C:** liver section from mice given ethyl acetate fraction (200 mg/kg) + CCl₄, **D:** liver section from mice given ethyl acetate fraction (400 mg/kg), **E:** liver section from mice given aqueous fraction (200 mg/kg) + CCl₄, and **F:** liver section from mice that received aqueous fraction (400 mg/kg) + CCl₄.

Protective effect of extract and fractions on human HepG2 cell lines

The results revealed that CCl₄ toxicity decreased cell viability to 39.23 %, when compared to 99.9 % viability in normal control, indicating that CCl₄ was very toxic to the cells. However, the methanol extract and its fractions restored the viability of HepG2 cells in a dose-dependent fashion, as shown in Figure 3. At the highest dose of 100 µg/mL, the hepatoprotective effects of the ethyl acetate fraction and methanol extract (84.5 and 74.2 %, respectively) were better than that of the ethyl acetate fraction (74.2 %). These results are presented in Figure 2.

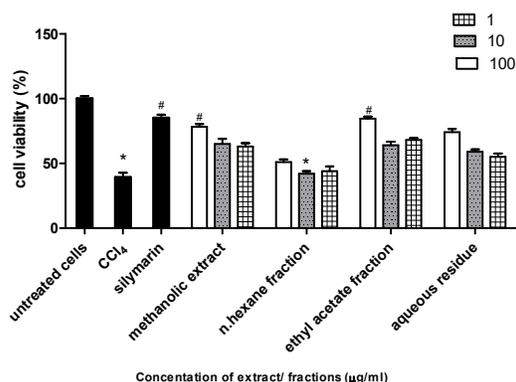


Figure 3: Effect of methanol extract of *F. carica* and its fractions on viability of CCl₄-treated HepG2 cells

DISCUSSION

The metabolism and detoxification of drugs or xenobiotics in the liver generate highly toxic free radicals as by-products. These free radicals bind to macromolecules and cause oxidative damage to hepatocytes, leading to hepatic injury. Hepatoprotective compounds obtained from plant sources are of great interest to researchers because they have no side effects, unlike synthetic drugs. The protective effect of plants is due to the presence of polyphenols and flavonoid compounds which have antioxidant properties due to their free radical-scavenging capacities [13].

Carbon tetrachloride is used to induce hepatotoxicity in experimental animals. It is a reliable method for screening the hepatoprotective effects of drugs [14]. The drug CCl₄ is a biologically inactive and stable molecule. However, it is metabolized by cytochrome p450 system to yield a toxic metabolite. Initially, CCl₄ is converted to trichloromethyl free radical, and then in the presence of oxygen, trichloromethyl free radical is converted to trichloromethyl peroxide. These

free radicals cause oxidative stress, destruction of plasma membrane and damage to liver tissues [15].

The magnitude of hepatic damage and protection are determined by estimating the serum levels of liver biomarkers such as ALT, AST, bilirubin, ALP and protein [16]. Liver biomarkers are present in the mitochondria of hepatocytes. However, CCl₄ damages the hepatocyte membrane, leading to loss of structural integrity and leakage of liver enzymes from the mitochondrion into the blood circulation. Significant increases in the levels of liver biomarkers i.e. ALT, AST, ALP and total bilirubin were observed in the group of mice treated with CCl₄. However, these increases were reversed by treatment with plant extract and fractions at doses 200 and 400 mg/kg. Higher significant hepatoprotective effects were produced by treatment with extract and fractions at the higher dose of 400 mg/kg body weight, indicating that the hepatoprotective effect of *Ficus carica* was dose-dependent. The observed hepatoprotective effects of *Ficus carica* extract and fractions are most likely due to the presence of bioactive compounds such as phenolics and flavonoids which have been reported to be present in the plant [17]. Thus, it can be reasonably stated that the CCl₄-induced hepatic damage was reversed by phenolics and flavonoids present in the extract and its fractions. The extract fractions also increased serum protein levels which were decreased when animals were administered CCl₄.

Carbon tetrachloride (CCl₄) intoxication results in the formation of highly reactive free radicals. The biological system protects itself from free radicals with the help of endogenous antioxidant enzymes such as reductases, CAT, SOD and GPx. Moreover, the body can be protected via intake of exogenous and natural antioxidant compounds from plants. In this study, the effects of the extract and its fractions on antioxidant defenses in CCl₄-toxified mice were determined by assaying the activities of CAT, SOD and GPx.

Hydrogen peroxide is converted into water and molecular oxygen by the endogenous antioxidant enzyme CAT [18]. In this study, administration of *Ficus carica* extract and fractions enhanced the activity of CAT, resulting in mitigation of CCl₄ toxicity in hepatocytes. Superoxide dismutase (SOD) converts the highly reactive superoxide anion O₂⁻ to molecular oxygen and hydrogen peroxide. The results obtained in the present study revealed that CCl₄ administration suppressed the activity of SOD. This is

consistent with previous reports. However, treatment with *Ficus carica* extract and fractions enhanced the activities of the antioxidant enzymes. The endogenous antioxidant enzyme GPx neutralizes free radicals, thereby overcoming the oxidative stress imposed by ROS. In this study, administration of *Ficus carica* reversed the CCl₄-induced decreases in GPx activity. Histopathology aids in the assessment of the liver damage and hepatoprotective effects of drugs. Several studies have reported structural changes in the liver morphology caused by CCl₄ [19].

In vitro systems based on cultured immortalized hepatoma cell lines are widely used to study the effects of toxic substances on hepatocytes [10]. The HepG2 cell lines are extensively used, when compared to primary culture of hepatocytes from animals and man, because it is easy to subject them to cryopreservation and culture. Moreover, the biochemical and morphological characteristics of normal hepatocytes are retained in HepG2 cell lines [21].

The hepatoprotective effects of several plant products against CCl₄-induced lesions have been established in studies using HepG2 cell lines. In the present study, MTT assay was applied to determine the hepatoprotective effects of methanol leaf extract of *Ficus carica* and its fractions on HepG2 cell line. The tetrazolium dye (MTT) is often used to determine the viability and metabolic characteristics of cells based on the conversion of the yellow tetrazolium bromide salt to red formazan crystals by mitochondrial succinate dehydrogenase.

CONCLUSION

The results of the present study indicate that the leaves of *Ficus carica* exert strong protective potential against oxidative stress induced by CCl₄. The methanol leaf extract of *Ficus carica* and its fractions reverses CCl₄-induced liver damage, thereby preventing leakage of liver enzymes into blood circulation, and maintaining hepatocyte structural integrity. The extract also restored the activities of liver antioxidant enzymes. The results also reveal that the ethyl acetate fraction produced very high hepatoprotective effect which might be due to the presence of high levels of free radical-scavenging phenolic and flavonoid compounds. Thus, *Ficus carica* leaves may serve as an alternative to the natural hepatoprotective drug, silymarin.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. SH and HG carried out the study; RQ participated in plant collection and identification, while MG and SH designed the experiments. SH wrote the manuscript. SMSN and MG supervised the work. All authors read and approved the final manuscript.

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