

Hepatoprotective Activity of *Cichorium intybus* L. Leaves Extract Against Carbon Tetrachloride Induced Toxicity

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Abstract

The effects of different concentrations of the hydroalcoholic extract of dried powdered leaves of *Cichorium intybus* L., on CCl₄-induced hepatotoxicity *in vivo* in rats and CCl₄-induced cytotoxicity in isolated rat hepatocytes were investigated. Rats received different concentrations of the extract by i.p. injection for 3 consecutive days before the injection of (3ml/kg) CCl₄ (i.p.). Twenty four h after CCl₄ injection the animals were sacrificed and the livers were dissected for biochemical and histopathological studies. The results showed that the *Cichorium intybus* extract could protect the liver from CCl₄-induced damages with doses of 50 and 100 mg/kg, but concentrations higher than 200 mg/kg were less effective. For *in vitro* studies, the extract were added to the suspension of freshly isolated rat hepatocytes incubated in Krebs-Henseleit buffer under a gas flow of 95% O₂ and 5% CO₂, 20 minutes before the addition of 10 mM of CCl₄. The extract with concentrations of 60 to 600 µg/ml protected the cells against CCl₄-induced cytotoxicity, but concentrations of ≥ 1.5 mg/ml and higher increased the CCl₄-induced cytotoxicity. The *Cichorium intybus* extract itself was toxic towards isolated hepatocytes in concentrations above 3.6 mg/ml. The results of the present study therefore supported the traditional believes on hepatoprotective effect of the *Cichorium intybus* extract, however, high concentrations were hepatotoxic.

Keywords: *Cichorium intybus*; CCl₄; liver; hepatoprotective; hepatocytes.

Introduction

Cichorium intybus, known as “chicory” has been implemented in folk medicine from north Africa to south Asia for several hundred years. Aquadistillate of aerial parts of *Cichorium intybus* (Aragh-e-Kasni) is used to purify blood and liver disease in different parts of Iran. In the Persian folk medicine, the seeds and leaves of the plant have been considered to be hepatoprotective and

blood purifier (1, 2). The seeds of the plant are used in hepato-biliary disorders in Ayurvedic medicine. The antihepatotoxic activity of the seeds and roots of *cichorium intybus* have been previously reported (3, 4). The present study was performed to find out whether hydroalcoholic extract of *Cichorium intybus* leaves demonstrate any hepatoprotective activity against CCl₄-induced liver damage *in vivo* (in rats) and or in freshly isolated rat hepatocytes. And also to compare, its hepatoprotective effect to that of silymarin which is known to be hepatoprotective against CCl₄- or acetaminophen-induced liver damages (5, 6).

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Materials and methods

Plant Material and Chemicals

Fresh leaves of *Cichorium intybus* were collected during March 2002 from rural areas around Shiraz, Iran, and authenticated by botanist at the Botany Department, Shiraz University, Shiraz, Iran. The plant was dried at the room temperature.

CCl₄ and EGTA were purchased from Sigma-Aldrich Chemical Company, Collagenase from Clostridium Histolyticum form Worthington Company, HEPES (4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid) and BSA (Bovian Serum Albumin) form Boehringer-Mannheim, and EGTA (Ethylene Glycol-bis(β-aminoethyl Ether) -N,N,N',N'-Tetraacetic Acid) from Sigma Chemical Company. Silymarin extract was a gift from Dr. Amanzadeh, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

Animals

Male Sprague-Dawley rats (200-250 g) were obtained from the Laboratory Animals Research Center of Shiraz University of Medical Sciences. The rats were maintained under controlled temperature, 12 h light/12 h dark conditions for one week before the start of the experiments. They had access to standard laboratory chow and tap water ad libitum.

Preparation of Extracts

Fifty grams of dried powdered leaves were macerated in 70% EtOH (100 ml) at room temperature for 48 h. The extract was filtered and concentrated under reduced pressure and low temperature (40°C) on a rotary evaporator to a concentration of 130 mg/ml. The yield of dried extract was 260mg/1g dried leaves.

In vivo hepatoprotective activity studies

Sixty three rats were randomly divided into 9 groups of seven animals. Group I received saline (10 ml/kg, i.p) as normal control; group II received CCl₄/olive oil (1:1, 3 ml/kg, i.p) as treated control; group III received silymarin extract (25 mg/kg, i.p) as the standard reference; groups IV, V and VI received the extract with doses of 50, 100 and 200 mg/kg, respectively, for 3 consecutive days. Groups VII, VIII, and

IX received 50, 100, or 200 mg/kg of the extract respectively by i.p. injection for 3 days and CCl₄/olive oil (1:1, 3 ml/kg, i.p.) on the third day. Twenty four h after CCl₄ injection animals were anaesthetized by pentobarbital injection (50 mg/kg) and blood was collected from the vena cava, and the serum was separated for subsequent use for different enzyme measurements. The rats were then decapitated and the livers were carefully dissected and cleaned of extraneous tissues. Part of the liver tissue was immediately transferred to 10% formalin for histopathological assessments.

Measurement of ALT, AST and ALP in serum of rats

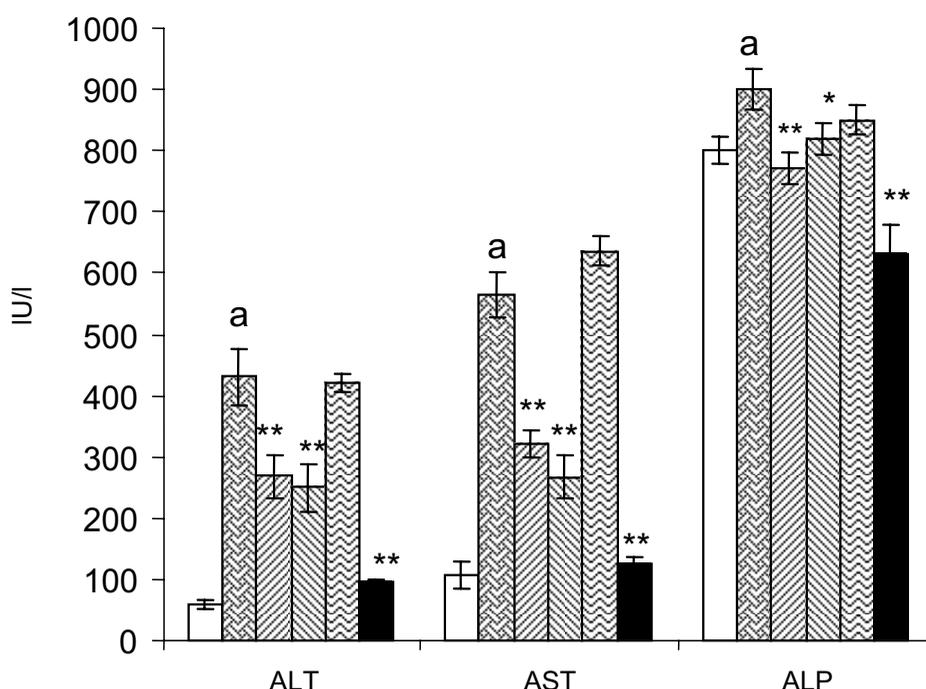
Bioconstandard kits and DAX-48 autoanalyzer were used to measure alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) activities in serum according to the methods of Wilkinson et al (1972), and Bessay et al (1946), (7, 8).

Histopathological Studies

The livers were removed from the animals and the tissues were fixed in 10% formalin for at least 24 h. Then, the paraffin sections were prepared (Automatic tissue processor, Autotechnique) and cut into 5 μm thick sections using a rotary microtome. The sections were then stained with Haematoxylin-Eosin dye and studied for histopathological changes, i.e. necrosis, fat changes, ballooning degeneration, and lymphocyte infiltration. Histological damages were scored as: 0: absent; +: mild; ++: moderate; and +++: severe.

In vitro hepatoprotective activity studies

Hepatocytes were isolated from Sprague-Dawley rats by collagenase perfusion technique as described previously (9). Hepatocytes (1x10⁶ cells/ml) were suspended in Krebs-Henseleit buffer (pH:7.4), containing 12.5 mM HEPES and kept in rotating, round bottomed 50 ml flasks at 37°C under a continuous flow of 95% O₂ and 5% CO₂. The cells were let to get adapted with incubation condition for 20 min before the addition of compounds to the incubation mixture. The cells were then exposed to different concentrations of the Chicory extract with and without CCl₄



□ Normal ▨ CCl₄ ▩ CCl₄+chicory50 ▪ CCl₄+chicory100 ▫ CCl₄+chicory200 ■ CCl₄+Silymarin
Figure 1. The protective effect of different doses of the *Cichorium intybus* extract on CCl₄ induced rat hepatic enzyme level elevation. Rats were injected (i.p) with determined concentrations of the *Cichorium intybus* extract for 3 consecutive days before injection of 3 ml/kg CCl₄. The *Cichorium intybus* extract alone did not increase the levels of the enzymes. Level of enzymes were measured in rat serum according to what explained in materials and methods. Values were demonstrated as mean ± SD of 7 rats per group.

a: Significantly different from control group (p<0.05).

*: Significantly different from CCl₄-treated group (p<0.05).

** : Significantly different from CCl₄-treated group (p<0.01).

chicory.: *Cichorium intybus*

10 mM (CCl₄ / ethanol, final concentration 1%) and aliquots of the cells were taken out at different time points (30, 60, 120 and 180 min) for determination of cell viability. Cell viability was assessed by Trypan Blue Exclusion test.

Statistical Analysis

The data obtained from *in vivo* studies were analyzed by student's "t" test and one-way ANOVA followed by Dunnett post test. For isolated hepatocyte studies, the results were shown as mean ± standard deviation (SD) of triplicate samples. The differences between the control and experimental groups considered significant if $p < 0.05$.

Results and Discussion

In vivo hepatoprotective activity

Administration of CCl₄ to rats caused a significant elevation in serum activities of ALT, AST and ALP after 24 h. Treatment of rats

with 50 or 100 mg/kg doses of the *Cichorium intybus* extract (i.p.) markedly prevented CCl₄-induced elevation of serum ALT, AST and ALP. However, 200 mg/kg of the extract did not prevent elevation of the enzymes (Figure 1). Serum bilirubin (total and direct) levels were also significantly enhanced by CCl₄ treatment but only total bilirubin was remarkably reduced by pretreatment with 50 mg/kg of the extract (data not shown).

Silymarin with a dose of 25 mg/kg also significantly prevented CCl₄-induced elevation of serum ALT, AST and ALP activities (Figure 1).

Histopathological examinations of the liver sections of the rats treated with CCl₄ showed focal necrosis, fatty changes, ballooning degeneration and infiltration of lymphocytes around the central veins. Necrosis, which is a more severe form of injury, was markedly prevented by pretreatment with 50 and 100 but not 200 mg/kg doses of the extract (Table 1).

Table 1. Effect of *Cichorium intybus* extract on histopathological damages induced by CCl₄ injection in rats.

Groups	Microscopic Observation			
	Necrosis	Fatty changes	Hepatocytes deformation	Lymphocyte infiltration
Control	+	0	0	+
CCl ₄ (3 ml/kg)	+++	+++	++	+++
Chicory (50mg/kg)	+	0	0	+
Chicory (100mg/kg)	+	0	0	+
Chicory (200mg/kg)	++	0	0	+
CCl ₄ + Chicory (50mg/kg)	++	+	+	+
CCl ₄ + Chicory (100mg/kg)	++	+	+	+
CCl ₄ + Chicory (200mg/kg)	++	+++	++	+++
Silymarin (25mg/kg)	+	0	0	+
CCl ₄ + silymarin (25mg/kg)	+	0	0	+

0: absent +: mild ++: moderate +++: severe

Rats were injected (i.p) with determined concentrations of *Cichorium intybus* extract for 3 consecutive days before injection of 3 ml/kg CCl₄. Histopathological damages were assessed as explained in material and methods.

***In vitro* hepatoprotective activity**

Carbon tetrachloride was toxic towards isolated rat hepatocytes in a dose- and time-dependent manner, with a TC₅₀ of about 10 mM, for 1 h (data not shown).

The results presented in Table 2 indicated that the preincubation of hepatocytes with concentrations between 60 to 600 µg/ml of the Chicory extract for 20 minutes protected hepatocytes against CCl₄-induced cytotoxicity. The protective effect of the Chicory extract was

dose-dependent and concentrations higher than 1 mg/ml did not show any protective effect against CCl₄ induced cytotoxicity. Silymarin also significantly protected hepatocytes against CCl₄ cytotoxicity with a dose of 200 µg/ml (Table 2). Furthermore, the *Cichorium* extract itself caused cytotoxicity towards hepatocytes with concentrations higher than 3 mg/ml and caused more than 90% cell death at 3 h of incubation with a concentration of 3.5 mg/ml (Table 3).

CCl₄ is metabolized by microsomal CYP450

Table 2. Protective effect of *Cichorium intybus* extract against CCl₄-induced cytotoxicity in isolated rat hepatocytes.

	% Cytotoxicity Time (min)			
	30	60	120	180
Control	18±2	20±3	25±3	28±4
CCl ₄ (10 mM)	30±3 ^a	49±3 ^a	66±4 ^a	82±4 ^a
+ chicory (60 µg/ml)	24±3**	35±3*	52±3**	72±3*
+ chicory (120 µg/ml)	23±2**	38±2*	58±3*	68±4*
+ chicory (240 µg/ml)	22±2**	33±2**	42±3**	52±4**
+ chicory (360 µg/ml)	23±2**	30±2**	45±3**	48±3**
+ chicory (480 µg/ml)	22±2**	35±3*	38±2**	48±3**
+ chicory (600 µg/ml)	27±2	35±2*	55±3*	65±4*
+ chicory (1 mg/ml)	27±2	43±3**	60±3*	78±4
+ chicory (1.2 mg/ml)	26±2*	41±3**	65±3	83±4
+ chicory (1.5 mg/ml)	32±3	47±3	70±3	89±5*
+ silymarin (200 µg/ml)	25±3**	32±3**	43±3**	63±4*

Isolated rat hepatocytes (10⁶ cells/ml) were incubated in Krebs-Henseleit buffer, pH=7.4 under a flow of 95% O₂ and 5% CO₂. The cells were preincubated with 60 µg- 1.5mg/ml chicory extract for 20 min before the addition of CCl₄. Cell viability was assessed by trypan blue exclusion test. Results were demonstrated as mean ± S.D. of at least 3 different experiments.

a: Significantly different from control group (p<0.05).

*: Significantly different from CCl₄-treated cells (p<0.05).

** : Significantly different from CCl₄-treated cells (p<0.01).

Table 3. Effect of *Cichorium intybus* extract in freshly isolated rat hepatocytes

	% Cytotoxicity Time (min)			
	30	60	120	180
Control	18±2	20±3	25±3	28±4
chicory (120 µg/ml)	18±2	21±2	24±3	25±3
chicory (240 µg/ml)	19±2	22±2	24±3	27±3
chicory (600 µg/ml)	17±2	22±2	23±2	27±3
chicory (1.2m g/ml)	18±2	21±2	23±3	28±3
chicory (2.4 mg/ml)	18±2	21±2	24±2	30±3
chicory (3.6 mg/ml)	17±3	25±3*	35±3**	93±5**
chicory (4.8 mg/ml)	27±3**	42±4**	100**	100**

Freshly isolated rat hepatocytes (10^6 cells/ml) were incubated with different concentration of *Cichorium intybus* extract in the absence of CCl_4 . Trypan blue exclusion was expressed as percentage of the total activity. Results were demonstrated as mean \pm S.D. of four experiments. (* $p < 0.05$ ** $P < 0.01$): Significantly different from control for the corresponding time points.

in the liver to a highly reactive trichloromethyl free radical ($\text{C}^\circ\text{Cl}_3$) which can start a chain of reactive free radical formation resulting in peroxidation of lipids and damage to proteins and components of the cell leading to cell lyses (10, 11). Therefore, it is used for induction of experimental liver damage *in vivo* or in isolated liver cells for hepatoprotective studies of drugs and medicinal plants (12, 13).

In the present study, CCl_4 caused histopathological damage to the rat liver and increased the serum levels of ALT, AST and also ALP, and bilirubin, also caused cytotoxicity in isolated rat hepatocytes. The Chicory leave extract with concentrations of 50 and 100 mg/kg protected the rat liver against CCl_4 -induced damages and lowered the serum ALT, AST, and ALP, *in vivo*, and protected isolated rat liver cells against cytotoxicity of CCl_4 . Since toxicity of CCl_4 is believed to be due to free radical formation and oxidative stress, therefore protective effect of the Chicory extract is possibly by one or several mechanisms such as: inhibition of cytochrome P450 activity responsible for metabolism of CCl_4 to reactive free radicals; antioxidant effects; or scavenging free radicals responsible for cell damage. The Chicory extract with a dose of 200 mg/kg did not protect rat liver against CCl_4 damage. CCl_4 also caused cytotoxicity in isolated rat hepatocytes with high concentrations, but its mechanism of cytotoxicity is unclear.

Therefore, it is possible that the Chicory extract with concentrations above 200 mg/kg have imposed further insults to the liver and its toxic effect overcame its protective effect against CCl_4 .

Pervious studies of Gadgoli and Mishra (14), and Ahmad et al., (15) also showed that total aqueous and alcoholic extract of *Cichorium intybus* seeds reduced the levels of ALT and AST in paracetamol or carbon tetrachloride induced hepatotoxicity, which is in full accordance to results of the present study. It has also been reported that aqueous and alcoholic extracts of *Cichorium intybus* seeds and root or root callus protected rat liver against carbon tetrachloride induced hepatocellular damage (4).

However, to clarify the hepatoprotective mechanism(s) and also to determine the active component(s) of the Chicory extract we need further investigation. In conclusion, the results of the present study suggest that the Chicory extract could prevent oxidative liver damage whereas high concentrations could cause damage to liver.

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