Anti-Hepatotoxic Effect of the Methanolic Anstatica Hierochuntica Extract In CCl₄-Treated Rats

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Abstract

The study was investigated the hepatoprotective activity of methanolic extract of Anstatica hierochuntica using carbon tetra chloride (CCl₄)-induced hepatotoxicity in rats. The levels of liver enzymes glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, alkaline phosphatase, total protein, total and direct bilirubin, in addition to Superoxide dismutase, glutathione peroxidase activities, glutathione malondialdehyde and total antioxidant status levels were evaluated in experimental rats (with or without CCl₄-induced hepatotoxicity) following intake of 100 mg/kg p.o alcoholic Anstatica hierochuntica extract by using standard procedures compared with standard silymarin at a dose of 100 mg/kg p.o. Results showed that methanolic extract at a dose level of 100 mg/kg had a significant decrease (p<0.05) in activities of serum liver enzymes, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, alkaline phosphatase, total bilirubin and protein were significantly decreased in rats treated with CCl₄ after 4 weeks compared to that of silymarin group in addition to the methanolic extract had antioxidant activity through decreasing activities of superoxide dismutase and glutathione peroxidase to levels in control rats group. Histopathology of a liver tissue of the animals treated with the extract was also studied to monitor the liver status. The liver biopsy of all experimental rat groups treated with the methanolic Anstatica hierochuntica extract showed significant restoration of the normal histomorphologic pattern of liver cells. From the above results, it is concluded for the first time that methanolic Anstatica hierochuntica extract offers protective effect against CCl₄-induced hepatotoxicity in experimental rats.

تأثير الوقائي للكبد للمستخلص الكحولي نبات كف مرير في القنارن المعاملة برابع كلورد الكربون

الخلاصة

تضمنت الدراسة تأثير المستخلص الكحولي نبات كف مرير لمعالجة تسمم الكبد المستحدث في القنارن المعاملة برابع كلورد الكربون. تم قياس مستويات إنزيمات الكبد الدافئة لمجموعة الأمين الأمبارتية، وتقلة لمجموعة الأمين الأنثنيبة، وزمن الفوسفاتي دقاعدي و البروتين الكلوي والبروتينات الكلوي والمتباينة. إضافة إلى مستوى الكولتانول، والكولتانولين إضافة إلى نسبة الكولتانولين إضافة إلى نسبة الكولتانولين ونسبة الكولتانولين إضافة إلى نسبة الكولتانولين. نتائج المستخلص الكحولي بالرابع كلورد الكربون بعد تناولها جرعات 100 ملغ./كم/كغ من وزن الفار من المستخلص الكحولي لنبات كف مرير ومقارنةها مع مستوى السيكلارين القاسي بجرعة 100 ملغ./كم/كغ من وزن الفار. أظهرت النتائج أن المستخلص الكحولي لنبات له تأثير معنوي 0.05 على خفض مستويات

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Introduction

The liver is a major detoxifying organ in vertebrate body, which involves intense metabolic activities. Certain toxic chemicals and medicines can cause liver damage, which has been recognized as a toxicological problem. Amp experimental and epidemiological studies support the involvement of oxidative stress in the pathogenesis and progression of several chronic diseases (1). Oxygen, indispensable for maintaining life, sometimes becomes toxic, resulting in the generation of most aggressive agents such as reactive oxygen species (ROS). Aerobic organisms employ a battery of defense mechanisms such as antioxidant enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) to prevent or mitigate oxidative tissue damage (2). SOD removes the superoxide radical to prevent formation of the hydroxyl radical. Catalase deals effectively with a large amount of hydrogen peroxide generated in peroxisomes. GPx is capable not only of utilizing hydroperoxides but also of metabolizing hydrogen peroxide in both the cytosolic and mitochondrial compartments. When the liver cell plasma membrane is damaged, many of the enzymes normally located in the liver cell cytosol are released into the blood stream. Their estimation in the blood is a useful quantitative marker of the extent and type of hepatocellular damage (3). Perturbation of the GSH status of a biological system has been reported to increase the lipid peroxidation (4). Thiobarbituric acid reactive substances (TBARS) are produced as byproducts of lipid peroxidation that occurs in the hydrophobic core of biomembranes (5). At other sites, intake of compounds that induce antioxidant enzyme activity or scavenging of free radicals prevents oxidative damage (6). However, herbal medicines are known to play an important role in the treatment of various ailments, including hepatopathy (7). Many traditional practitioners have claimed that numerous medicinal plants and their formulations can be effectively used for the alleviation of different types of liver diseases (8). But most claims are anecdotal and very few have received adequate medical and scientific evaluation. Anastatica hierochuntica was widely used as medicinal plant either by itself or in combination with other herbs. The whole plants of Anastatica hierochuntica is commonly called “Kaff maryam” or “Rose of Jericho”, which is a winter annual plant of the Sahara-Arabian deserts, was prescribed in Egyptian folk
Anti-hepatotoxic effect of the methanolic Anastatica hierochuntica extract in CCl<sub>4</sub>-treated rats.

The present investigation was carried out to examine whether the methanolic extract might also have a protective effect against carbon tetrachloride (CCl<sub>4</sub>) induced hepatotoxicity in vivo. The exact mechanism of hepatotoxicity of CCl<sub>4</sub> is unclear, most probably resulting from a toxic intermediary that binds covalently to hepatocytes and causes a centrilobular hepatic necrosis. It has been established that CCl<sub>4</sub> is accumulated in hepatic parenchyma cells and metabolically activated by cytochrome P-450 dependent monooxygenases to form a trichloromethyl free radical (CCl<sub>3</sub>). The latter alkylates cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids in the presence of oxygen to produce lipid peroxides leading to liver damage.

**Materials and Methods**

**Reagents.**

Reagents for antioxidant enzyme assays were purchased from Sigma Chem. Co. (USA). Kits RANSEL (RS504), RANSOD (SD125) and TAS (N 2332) for determination of the activity of glutathione peroxidase and superoxide dismutase as well as total antioxidative status (TAS), respectively, were purchased from Randox Laboratories Ltd. (UK). Silymarin as the reference hepatoprotective drug was obtained from Extrasynthese (France). All other chemicals were of reagent grade and were used without further purification.

**Plant materials**

Samples of *Anastatica hierochuntica* plant were purchased from Iraqi local market in Baghdad. The plant material was authenticated by a taxonomist professor Ali Al-Mosowi at the Department of Botany College of science, University of Baghdad, Iraq. The samples were washed with clean tap water to remove dirt on the leaves. The dried plant material was manually powdered and the powder kept in polyethylene bags until used.

**Preparation of methanolic extract**

The powdered whole plant (100 g) kept in a thimble was extracted with 200 ml 70% methanol in a soxhlet extractor. The extract was concentrated in vacuum at 60 °C using a rotary evaporator. To evaporate the remaining solvent, the extract was kept in an oven at a temperature of 40-50 °C for 8 hours. The alcoholic extract (250 mg/kg) was formulated as suspension in 1% w/v tracaganth gum and used for the study. Silymarin was used as a standard drug.

**Animals**

Male Wister strain albino rats (40) weighing 150 – 200 g and they were maintained under standard environmental condition (temperature 25 – 28 C and 12 hr light/dark cycle) and allowed access to standard laboratory feed and water ad libitum. The rats were allowed to acclimatize to the laboratory condition for a week before they were used for the experiment. Ethical approval for the use of the animals was obtained from the institutional committee constituted for the purpose.

**Acute toxicity studies**

Acute oral toxicity was performed according to Ecobichon DJ (11). The male albino rats were fasted over night provided only water, after which the alcoholic extract of *Anastatica hierochuntica* was administered by
gastric intubation to the relevant animals orally at the dose of 5 mg/kg body weight. The animals were then observed for 14 days. When mortality was observed in 2 or 3 animals, the dose administered was recorded as a toxic dose. But when mortality was observed in one animal, then the same dose was repeated again for confirmation. However, if mortality was not observed, the procedure was repeated for further higher doses such as 50, 300 and 2,000 mg/kg body weight. Toxic symptoms for which the animals were observed for 72 hr include some behavioral changes, locomotion, convulsions and mortality.

**Hepatoprotective activity**

Hepatotoxicity was evaluated as previously described (12). A total of 40 albino rats were divide 4 groups of 10 animals each and treated as follows: **Group I (Control):** received subcutaneous administration of 1% w/v of gum tracaganth at the dose of 1ml/kg /day per oral for 14 days.

**Group II:** received subcutaneous administration carbon tetra chloride (CCl\(_4\) at a dose of 0.1 ml/kg/day) of body weight i.p for 10 days.

**Group III (test):** served as test and received alcoholic extract of *Anastatica hierochuntica* (100mg/kg p.o) daily for 14 days along with CCl\(_4\) subcutaneous for 10 days.

**Group IV (standard):** received silymarin (100 mg/kg) per oral for 14 days along with CCl\(_4\) subcutaneously for 10 days. At the end of the 14th day, the blood was collected from all the animals from the rectero orbital plexus and the serum was separated by centrifugation at 2000 rpm for 10 min. The serum was then assayed for hepatic marker enzymes, serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), total and direct bilirubin and total protein, glutathione (GSH), lipid peroxidation marker such as malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx) and finally total antioxidant status (TAS).

**Assessment of biochemical parameters**

The estimation of S.GOT and S.GPT was based on the reference method described in International Federation of Clinical Chemistry (13). The reagent supplied in the kits (Randox Diagnostic Kits) were reconstituted and mixed with the serum. S.GOT and S.GPT were measured at 340 nm and expressed as IU/L. Serum ALP was estimated by mixing the reagent (p-nitrophenyl phosphate, magnesium, buffers and stabilizers) with the serum and measuring the absorbance at 405 nm (14). The value obtained was expressed as IU/L. Total protein was measured according to the method of Bradford using bovine serum albumin as a standard (15). The absorbance of the solution was then measured at 555 nm and the estimated total protein was expressed as gm/dl. Total and direct bilirubin were estimated by the method of Jandrassik and Grof at 546 nm and expressed as mg/dl (16). The activities of hepatic marker enzymes (GPx and SOD) and biochemical parameter TAS were assayed in blood using standard kits: RANSEL kit (17), RANSOND kit (18) and TAS kit (19) for determination of the activity of glutathione peroxidase and superoxide dismutase as well as total antioxidative status (TAS) respectively. Colorimetric estimation of reduced glutathione (GSH) in blood was performed as described by Beutler (20). The quantitative
measurement of lipid peroxidation was done by measuring the concentration of thiobarbituric acid reactive substances (TBARS) in serum using the method of Ohkawa et al. (21). The amount of malondialdehyde (MDA) formed was quantitated by the reaction with thiobarbituric acid and used as an index of lipid peroxidation. The results were expressed as nmol MDA/mg protein. Silymarin as the reference hepatoprotective drug was obtained from Extrasynthese (France). All other chemicals were of reagent grade and were used without further purification.

**Histopathological examination**

Animals were sacrificed by cervical dislocation and the blood was collected from the rectero-orbital plexus. The liver was removed, sliced and washed in saline and the pieces were preserved in 10% formosal (10% formaldehyde diluted with normal saline) to evaluate histopathological changes. Sections of pieces of the liver (about 4-6 mm in thickness) were processed and embedded in paraffin wax, stained with haematoxylin and eosin, mounted and observed under light microscope for histological changes (22).

**Data analysis**

As appropriate, measurements were carried out in triplicates and descriptive statistics (Mean ± SD) were used in presentation of the results. Data comparison was carried out using one way analysis of variance (ANOVA). At 95% confidence interval, 2-Tailed p-values less than 0.05 were considered to be significant.

**Results**

The marker liver enzyme levels are provided in the Table 1. There was significant increase in the levels of the marker enzymes (S. GOT, S. GPT and S. ALP) as well as total and direct bilirubin in the animals treated with CCl₄ when compared with the control animals. For the animals given the extract (100 mg/kg), the levels of these enzymes and bilirubin were relatively normal when compared with CCl₄ treated group (p<0.05). The serum total protein concentration of the CCl₄ treated group was significantly decreased to 3.1 ± 0.3 g/dl Vs. 5.5 ± 0.2 gm/dl in control animal group but administration of alcoholic plant extract increased the levels of total protein to 4.7±0.2gm/dl. The results in table 2 indicate that 14 days after CCl₄ administration, there was a significant increase in blood GPx (62.72 ± 9.5 U/ml) compared to the healthy group (35.15 ± 6.35U/ml). In the groups where sylimarin was given, the levels of GPx (34 ± 2.43 U/ml) were significantly lower (p < 0.05) than in the CCl₄-treated group (62.72±9.5 U/ml). The levels of SOD (140 ± 13U/ml) and TAS (0.38 ± 0.24mmol/L) were significantly reduced after intoxication with CCl₄ (p < 0.05). Acute exposure to a daily single intraperitoneal dose of CCl₄ for 10 days resulted in a severe decrease of GSH content in blood (38 ± 6.5 µmol/L) to (120 ± 16 µmol/L) of normal values. The depletion of GSH by CCl₄ was associated with an increase in lipid peroxidation as measured by the level of TBARS (2.53 ± 0.23*mmol MDA/mg protein). Intake of sylimarin or Anastatica hierochuntica extract for 7 consecutive days afforded different degrees of protection against such depletion. The most significant protection effect of plant extract was found at the levels of SOD and GPx (from 140 ± 13 to 430 ± 43 U/mL),
Anti-hepatotoxic effect of the methanolic Anastatica hierochuntica extract in CCl₄ treated rats.

for SOD and (from (62.72 ± 9.5 U/mL to (36.76 ± 2.30 U/mL for GPx. TAS (from 0.38 ± 0.24 to 0.62 ± 0.18 mmol L⁻¹ and MDA (from 6.53 ± 0.23 to 3.22 ± 0.35 nmol MDA mg⁻¹ protein (Table 2). The effect of Anastatica hierochuntica extract was quite close to that of sylimarin on the level of MDA bases and the TAS value. This protective effect of Anastatica hierochuntica methanolic extract was confirmed by histological examination as shown in Figure 1. Histological examination of animals in group I showed a normal hepatic architecture (Figure 1a). Animals in group II (CCl₄ treated) demonstrated severe hepatotoxicity as evidenced by profound steatosis, centrilobular necrosis and ballooning degeneration, Massive fatty changes, gross necrosis, broad infiltration of lymphocytes and of Kupffer cells around the central vein and loss of cellular boundary (Figure 1b). In group III and IV, the animal livers exhibited an almost normal architecture barring a little deformation of hepatocytes with pyknosis and clearing of cytoplasm (Figure 1c & 1d).

Discussion
Liver diseases remain as one of the serious health problems. However we do not have satisfactory liver protective drugs in allopathic medical practice for serious liver disorders. Herbal drugs play a role in the management of various liver disorders in addition to other natural healing processes of the liver (23). Previous studies have demonstrated the use of carbon tetrachloride to successfully induce hepatotoxicity in experimental animals (24). In experimental hepatopathy, the toxin carbon tetrachloride is biotransformed by cytochrome P-450 to produce the trichloromethyl free radical, which causes peroxidative degradation in the adipose tissue resulting in fatty infiltration of the hepatocytes. Trichloromethyl free radicals elicit lipid peroxidation of membrane lipids in the presence of oxygen generated by metabolic leakage from mitochondria. All these events culminate in loss of integrity of the cell membranes and damage of hepatic tissue (22). Histopathological examination of liver section of normal rats showed normal hepatic cells with cytoplasm and nucleus whereas CCl₄ treated group showed that the liver cells are intoxicated with CCl₄ and the normal architecture of the liver was completely damaged. The treatment of the rats with methanolic plant extract exhibited protection against liver damage by CCl₄ which is confirmed by the results of biochemical studies. The increase in the levels of transaminase reflects a clear indication of cellular leakage and loss of functional integrity of the cell membrane (26). Assessment of liver function can be made by estimating the activities of serum GOT and GPT, which are originally present in higher concentrations in cytoplasm. In hepatopathy, these enzymes leak into blood stream in conformity with the extent of liver damage (27). The elevated levels of marker enzymes (SGOT, SGPT, ALP), T.Bilirubin in treated CCl₄ rats in the present study corresponded to the extensive liver damage. A reduction in total serum protein (TSP) (Table 1) observed in the CCl₄ treated rats may be associated with the decrease in the number of hepatocytes which in turn may result into decreased hepatic capacity to synthesize protein and consequently decrease in the liver weight (Table 1). But, when the methanolic plant
Anti-hepatotoxic effect of the methanolic Anstatica hierochuntica extract In CCl₄ treated rats.

The methanolic extract was given along with CCl₄, the significant increase in TSP was observed indicating the hepatoprotective activity of extract and also accounting for the increase in the liver weight most probably through the hepatic cell regeneration. Treatment with the 100mg/kg methanolic plant extract of the plant significantly reduced the elevated liver enzymes and bilirubin level, indicating hepatoprotective action. Hepatotoxic effect of CCl₄ is due to oxidative damage by free radical generation and antioxidant property is claimed to be one of the mechanisms of hepatoprotective (28). In our work, the CCl₄-mediated hepatotoxicity was taken as the experimental model for liver injury. By estimating the activities of blood marker enzymes (GPx and SOD) and other biochemical parameters (TAS, GSH and MDA), an assessment of the liver function can be made. The changed activities of these liver marker enzymes observed in CCl₄-treated rats in our study correspond to the extent of liver damage induced by the toxin.

The tendency of these enzymes to return towards a near normal level in groups treated with sylimarin or Anstatica hierochuntica methanolic extract is a clear manifestation of their anti-hepatotoxic effect. Decline in GSH content in the serum of CCl₄-intoxicated rats, and its subsequent return towards near normally in sylimarin and in Anstatica hierochuntica extract treated rats reveal the antioxidant effect of the plant. Explanation of the possible mechanism underlying the hepatoprotective properties of the Anstatica hierochuntica extract includes the prevention of GSH depletion and destruction of free radicals (28). These two factors are believed to attribute to the hepatoprotective properties of Anstatica hierochuntica. Elevated levels of MDA observed in CCl₄-treated rats indicate excessive formation of free radicals and activation of the lipid peroxidation system, resulting in hepatic damage. The significant decline in the concentration of these constituents in the liver homogenate of sylimarin and Anstatica hierochuntica extract administered rats indicates anti-lipid peroxidative effect of Anstatica hierochuntica. In order to provide a better understanding of the possible role of the methanolic extract of Anstatica hierochuntica in the hepatoprotective effect observed in this study, we carried out a preliminary Phytochemical screening of the extract of the plant and found it to contain flavonoids and glycosides. Earlier report indicated that the flavonoids are phenolic compounds exert multiple biological effects, including antioxidant properties and free radical scavenging abilities (9). Over expression of fibrogenic cytokines as well as increased transcription and synthesis of collagen can be down regulated, at least in experimental models by the use of antioxidants and a study has demonstrated that natural phenolics inhibit satellite cell activation by perturbing signal transduction pathway and cell protein expression.

The co-administration of hepatoprotective agents may induce the hepatocytes to resist the toxic effects of carbon tetrachloride. Therefore, the protective activity of the extract may be due to its antioxidant property exerted by flavonoids in this plant.

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Anstatica hierochuntica extract In CCl₄ 
treated rats.


Table 1: Effect of oral administration of methanolic *Anastatica hierochuntica* extract on serum biochemical liver marker in CCl4 intoxicated albino rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Liver function markers</th>
<th></th>
<th>T.protein gm/dl</th>
<th>T.Bilrubin (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GOT(IU/L)</td>
<td>GPT(IU/L)</td>
<td>ALP(IU/L)</td>
<td></td>
</tr>
<tr>
<td>Vehicle control</td>
<td>10</td>
<td>60.2± 0.2</td>
<td>62.34± 3.11</td>
<td>195.7±15.4</td>
<td>5.5± 0.3</td>
</tr>
<tr>
<td>CCl4-treated</td>
<td>10</td>
<td>130.6± 4.3*</td>
<td>155.61±4.0**</td>
<td>315.2±14.0**</td>
<td>3.1± 0.3</td>
</tr>
<tr>
<td>Alcoholic extract</td>
<td>10</td>
<td>68.4± 0.54**</td>
<td>71.57± 0.71***</td>
<td>200.4±12.3**</td>
<td>4.70±0.2*</td>
</tr>
<tr>
<td>(100 m g/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silymarin</td>
<td>10</td>
<td>65.8± 3.1**</td>
<td>70.9± 4.2***</td>
<td>196.2±13.6**</td>
<td>5.4±0.20*</td>
</tr>
<tr>
<td>(100 mg/kg)</td>
<td></td>
<td></td>
<td></td>
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</table>

Table 2: Effect of oral administration of methanolic *Anastatica hierochuntica* extract on serum antioxidant enzymes (SOD and GPx), Glutathione, malondialdehyde, and total antioxidative status in CCl4 intoxicated albino rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>GPX U/ml</th>
<th>SOD U/ml</th>
<th>GSH µmol/L</th>
<th>TAS (mmol /L.)</th>
<th>MDA mmol /mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control</td>
<td>10</td>
<td>35.15 ±6.35</td>
<td>452 ± 20</td>
<td>120 ± 16</td>
<td>1.23 ± 0.13</td>
<td>3.83 ± 0.28</td>
</tr>
<tr>
<td>CCl4-treated</td>
<td>10</td>
<td>62.72 ± 9.5</td>
<td>140 ± 13</td>
<td>38 ± 6.5</td>
<td>0. 38 ± 0.24</td>
<td>6.53 ± 0.23*</td>
</tr>
<tr>
<td>Alcoholic extract</td>
<td>10</td>
<td>36.76 ± 2.3*</td>
<td>430 ±43***</td>
<td>98 ± 7.1**</td>
<td>0.62 ± 0.18*</td>
<td>3.22 ± 0.35*</td>
</tr>
<tr>
<td>(100 mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silymarin</td>
<td>10</td>
<td>34 ± 2.43**</td>
<td>398±27***</td>
<td>100 ± 6.4**</td>
<td>0.64±0.09*</td>
<td>3.32±0.25*</td>
</tr>
<tr>
<td>(100 mg/kg)</td>
<td></td>
<td></td>
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</table>

* Statistically significant difference vs. CCl4 group ($p < 0.05$). GPx – glutathione peroxidase, SOD – superoxide dismutase, TAS – total antioxidative status, GSH – reduced glutathione.
Anti-hepatotoxic effect of the methanolic Anstatica hierochuntica extract in CCl₄-treated rats.

Figure 1: Histological section of rat liver. Groups of rats: a) group 1, control (gum tracaganth for 14 days); b) group 2, CCl₄ for the 10th day); c) group 3, *Anastatica hierochuntica* extract (100 mg/kg for 14 days plus CCl₄, as above on the 10th day); d) group 4 sylimarin (100 mg/kg for 14 days plus CCl₄, as above on the 10th day).