Protection of *Citrullus colocynthis* Fruit Extracts on Carbon Tetrachloride-induced and Bacillus Calmette-Guerin plus Lipopolysaccharide-induced Hepatotoxicity in Mice

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**Abstract:** To investigate the hepatoprotective activities of the extracts from *Citrus colocynthis* (ECC), a native plant used as traditional Uigur Medicine on acute liver injury in mice. **Methods** The activities of ECC of petroleum ether (ECCPE), chloroform (ECCC), ethyl acetate (ECCEA), n-butyl alcohol (ECCBA), and water (ECCW) were evaluated in vivo using two experimental models, carbon tetrachloride (CCL4)- and bacillus calmette-guerin (BCG) plus lipopolysaccharide (LPS)-induced acute hepatotoxicity in mice. The contents of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum were determined and the liver histological examination was carried out, respectively. **Results** The pretreatment with ECC for 7 d obviously reduced the impact of CCL4 toxicity on the serum markers of liver damage, ECCEA and ECCC with a significant difference of AST ($P < 0.01, 0.05$, respectively) and ALT ($P < 0.05, 0.01$, respectively). The protective activity was reconfirmed against BCG + LPS-induced injury and the serum enzymatic levels were obviously elevated, for ECCEA and ECCC with a significant difference of AST ($P < 0.05, 0.01$, respectively) and ALT ($P < 0.01, 0.05$, respectively). **Conclusion** That ECCEA and ECCC are the potent hepatoprotective extracts that could protect liver against the acute injury, and this ability might be attributed to their hepatoprotective potentials.

**Key words:** bacillus calmette-guerin plus lipopolysaccharide; carbon tetrachloride; *Citrus colocynthis*; hepatoprotective activities; Uigur Medicine

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**Introduction**

*Citrullus colocynthis* (L.) Schrad. (Cucurbitaceae), also known as colocynth or bitter watermelon, is a common cucurbitaceous plant prevalent in arid areas of Mediterranean countries (Sebbagh *et al*, 2009; Adams *et al*, 2009). From the year 2009 to 2010, our research team cultivated the plant successfully in Xinjiang Changji Region of China (He *et al*, 2010; Hairula *et al*, 2011). *C. colocynthis* has been used medicinally since ancient times, mostly for constipation (Arena and Drew, 1980). In Saudi Arabia, the Arabs know that even treading barefoot on the squeezed fruit is enough to elicit its purgative action, presumably because the active compounds are readily absorbed percutaneously. In East Africa, the seed oil was applied to the skin by nomads in traditional medications (Habs, Jahn, and Schmahi, 1984). Many workers believed that the fruits of *C. colocynthis* possessed antitumor activity (Faust *et al*, 1958). It is reported that the fruits and seeds of *C. colocynthis* have the efficacy of analgesic and anti-inflammatory (Marzouka *et al*, 2010a), reversible antifertility activity (Chaturvedi, Mali, and Ansari, 2003), immunostimulating activity (Bendjeddou, Lalaoui, and Satta, 2003), antibacterial and antifungal activities (Marzouk, *et al*, 2009; 2010b; Abdel-Hassan, Abdel-Barry, and Mohammeda, 2000), and antidiabetic activity (Sebbagh *et al*, 2009).

It is commonly recognized that liver is a vital organ responsible for the metabolism of drugs and toxic chemicals. Meanwhile, liver is also prone to many...
diseases such as each type of hepatitis, alcohol damage, fatty liver, cirrhosis, cancer, and drug damage. Liver ailments remain one of the serious health problems (Joanne, Thanavaro, and ACNP-BC, 2011; Yang et al., 2012).

Our earlier preliminary investigation reported that the 70% ethanol extract of C. colocynthis (ECC) fruits caused a significant decrease in serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in male albino rats treated with carbon tetrachloride (CCl₄). The present investigation aims at determining the hepatoprotective activities of different ECC fractions against the experimental liver injury inflicted by two reliable hepatotoxins, CCl₄ and bacillus calmette-guerin (BCG) plus lipopolysaccharide (LPS), respectively.

Materials and methods

Chemicals and reagents
Carbon tetrachloride was purchased from Tianjin No.3 Chemical Reagent Factory (China) (batch No.: 061028). Ybarra extra virgin olive oil was produced by Aceites Ybarra, S.a. (Spain) (batch No.: 20101026). Bacillus calmette-guerin Vaccine for intradermal injection was produced by Shanghai Institute of Biological Products Co., Ltd. (China) (batch No.: 201011054-1). Sterilized water for injection was produced by Jiangsu Tianhe Disainuo Pharmaceutical Co., Ltd. (China) (batch No.: 20110523.2). Lipopolysaccharide (LPS) was purchased from Sigma Corporation of America (USA) (batch No.: L-2880). Sodium chloride injection (0.9%) was produced by Sinopharm Group Xinjiang Pharmaceutical Co., Ltd. (China) (batch No.: 20120210). Assay kits for serum AST and ALT were purchased from Biosino Bio-Technology & Science INC (China) (batch Nos.: 110861 and 111761). Bifendate Pills were produced by Beijing Union Pharmaceutical Factory (China) (batch No.: 101120108, 1.5 mg per pill). All other chemicals were of analytical grade.

Plant material

Citrullus colocynthis (L.) Schrad. was cultivated and collected in Manas County, Xinjiang, China (86°15′25″E, 44°18′14″N), in October 2009. The plant was identified by Prof. LIU Yong-min, Xinjiang Institute of Materia Medica (Urumqi, China), where a voucher specimen (No. 091121-09) has been deposited.

Preparation of plant extract

The dried and powdered fruits of C. colocynthis (10 kg) were extracted with 100 L of hydroalcoholic solution (70% ethanol and 30% water) by normal refluxing extraction for 1.5 h. The extraction process was repeated for three times. The combined extract was concentrated on a water bath and was vacuum distilled by rotary vacuum evaporator at 60 °C, yielding 2 L of water extract, successively partitioned in sequence with petroleum ether (6 × 200 mL), chloroform (6 × 200 mL), ethyl acetate (6 × 200 mL), and n-butyl alcohol (6 × 200 mL) leading to five extracts: petroleum ether (ECCPE, 550 g), chloroform (ECCC, 72 g), ethyl acetate (ECCEA, 45 g), n-butyl alcohol (ECCBA, 210 g), and water (ECCW) extracts.

Animals

Kunming mice (18—22 g) of both sexes were maintained under standard environmental conditions and had free access to food and water. All the animals were purchased from the Experimental Animal Centre of Centers for Disease Control of Xinjiang (China). Mice were kept in a specific room at (22 ± 1) °C and a 12-h light/dark cycle (lights on from 08:00 to 20:00), and provided with rodent chow and water ad libitum. The investigation conformed to the Guide for the Care and Use of Laboratory Animal published by the US National Institutes of Health (NIH publication No. 85 - 23, revised 1996).

CCl₄-induced hepatotoxicity

The protective activity of the extract against CCl₄-induced hepatotoxicity in mice was evaluated in the 7-day study. The animals were randomly divided into eight experimental groups of 10 mice each. The mice in the first group were served as normal control and received vehicle only (10 mL/kg distilled water) during the experiment. The mice in Group II were given 10 mL/kg distilled water for 7 d before CCl₄ intoxication and served as a hepatotoxicity control group. The mice in Groups IV—VIII were prophylactically ig treated for 7 d with five kinds of extract suspension (10 mL/kg, respectively). Before the last administration of vehicle or extract for 13 h, respectively, mice in Groups II—
VIII received an ip injection of CCl$_4$ (0.2 mL per mouse of 0.1% CCl$_4$ solution in olive oil). The mice in the control group were ip treated with an equal amount of olive oil alone. The animals were sacrificed 14 h after the CCl$_4$ and olive oil treatment, respectively. Blood samples were collected for the evaluation of the biochemical parameters.

**BCG + LPS-induced hepatotoxicity**

To study the effects of different extracts on BCG + LPS-induced liver injury, mice were randomly divided into eight groups with 10 animals per group. Group I: normal control group, Group II: toxic model control group, Group III: positive control group, and Groups IV—VIII: extracts pre-treatment groups. The mice in the pre-treatment groups were ig administered with different doses of different extracts (10 mL/kg), respectively, once a day for 10 d, while the mice in the control group were only given 10 mL/kg distilled water and mice in the positive control group were ig administered with 0.40 g/kg Bifendate Pills. The mice in each group were given a tail iv injection of BCG (0.2 mL) before the first administration. On the day 10, 15 h before the last administration, mice in Groups II—VI were given a tail iv injection of LPS in normal saline (0.2 mL per mouse, 8 μg), while the mice in the normal control group were tail iv injected equal amount of normal saline solution. The blood samples for the biochemical analysis were taken 16 h after the last administration.

**Measurement of AST and ALT**

The collected blood was centrifuged (3500 r/min, 10 min, 4 °C) and the serum was separated and stored at −20 °C before use. The enzyme activities were expressed as an international unit (U/L, μmol/L).

The AST activity in serum was determined by the method of Reitman and Frankel (1957). An aliquot of 1 mL of substrate (2 mmol/L α-ketoglutarate and 0.2 mol/L D, L-aspartate) was incubated with 0.2 mL of serum sample for 1 h at 40 °C. Then the reaction was clogged by addition of 1 mL of dinitrophenyl hydrazine (1 mmol/L). After 20 min, 10 mL of 0.4 mmol/L NaOH was added. The absorbance of the solution was measured at 505 nm after 30 min and distilled water served as a blank.

ALT activity in serum was determined by the method of Reitman and Frankel (1957). An aliquot of 1 mL of substrate and 0.2 mol/L L-alanine (for AST) was incubated with 0.2 mL of serum sample for 1 h at 40 °C. Then the reaction was clogged by the addition of 1 mL of dinitrophenyl hydrazine (1 mmol/L). After 20 min, 10 mL of 0.4 mol/L NaOH was added. The absorbance (A) of the solution was measured at 505 nm after 30 min and distilled water served as a blank.

**Histological examination**

For the histopathologic studies, mice were sacrificed under light ether anesthesia 16 h after the last dosage and the livers were removed and washed with normal saline. The liver tissues were fixed in 10% formalin, dehydrated with a sequence of ethanol solutions, and then embedded in paraffin. The tissue wax was cut into 5—6 μm sections, and fixed sections were then stained with both haematoxylin and eosin (HE). The histopathologic characteristics were observed and recorded under HPLAS—1000 colorized pathology image analyzer.

**Statistical analysis**

The quantitative data were expressed as X ± s. The statistical analysis of the data was performed with SPSS 11.5 statistical package (IBM, USA). All statistical comparisons were made by means of One-way ANOVA test followed by Tukey’s test. P < 0.05 was considered statistical significance while P < 0.01 was considered extreme significance. The IC$_{50}$ values were determined using a non-linear regression analysis of the dose-response curve.

**Results**

**Effects of ECC on CCl$_4$-induced mice liver injury**

The results on biochemical markers, commonly used for the evaluation of acute hepatic injury, in CCl$_4$-intoxicated mice were shown in Table 1. After single injection of CCl$_4$, the serum activities of AST and ALT enzymes in hepatotoxid model group (Group II) were significantly increased (P < 0.01) to (703.70 ± 301.93) and (579.85 ± 295.85) U/L, respectively, as compared with the normal control group (172.66 ± 22.32) and (51.26 ± 2.90) U/L. The elevated levels of these biochemical parameters clearly indicated the damage of hepatic cells. The mice in the positive control group were treated with the positive drug (Group III, 0.40 g/kg) for 7 d significantly reduced and the activities of serum AST and ALT were reduced.
Table 1  Protection of ECC extracts on CCl₄-induced elevation of AST, ALT, and liver coefficient in mice (X ± s, n = 10)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pretreatment dose / (g·kg⁻¹)</th>
<th>AST / (U·L⁻¹)</th>
<th>ALT / (U·L⁻¹)</th>
<th>Liver coefficient / %</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (normal control)</td>
<td>vehicle</td>
<td>172.66 ± 22.32</td>
<td>51.26 ± 2.90</td>
<td>4.64 ± 0.52</td>
</tr>
<tr>
<td>II (CCl₄ model control)</td>
<td>vehicle</td>
<td>703.70 ± 301.93</td>
<td>579.85 ± 295.85</td>
<td>4.96 ± 0.75</td>
</tr>
<tr>
<td>III (positive control)</td>
<td>0.40</td>
<td>414.89 ± 160.29</td>
<td>328.94 ± 164.86</td>
<td>4.77 ± 0.89</td>
</tr>
<tr>
<td>IV (CCl₄ + ECCW)</td>
<td>0.40</td>
<td>506.64 ± 196.09</td>
<td>458.38 ± 195.90</td>
<td>4.78 ± 0.59</td>
</tr>
<tr>
<td>V (CCl₄ + ECCBA)</td>
<td>0.40</td>
<td>516.49 ± 199.10</td>
<td>46.52 ± 117.91</td>
<td>4.72 ± 0.88</td>
</tr>
<tr>
<td>VI (CCl₄ + ECCEA)</td>
<td>0.40</td>
<td>339.58 ± 198.54</td>
<td>397.51 ± 109.46</td>
<td>4.67 ± 0.81</td>
</tr>
<tr>
<td>VII (CCl₄ + ECCC)</td>
<td>0.40</td>
<td>304.84 ± 150.84</td>
<td>305.96 ± 151.99</td>
<td>4.32 ± 0.79</td>
</tr>
<tr>
<td>VIII (CCl₄ + ECCPE)</td>
<td>0.40</td>
<td>416.79 ± 208.95</td>
<td>402.51 ± 98.84</td>
<td>4.62 ± 0.57</td>
</tr>
</tbody>
</table>

++P < 0.01 vs normal control group; *P < 0.05  **P < 0.01 vs CCl₄ model control group

(P < 0.05). Pre-treatment of animals with the different ECC (Groups IV—VIII, 0.40 g/kg) for 7 d obviously reduced the activities of serum AST and ALT, as compared with the CCl₄-treated group alone. The results of AST and ALT from ECCEA, ECCC, and ECCPE groups were significantly different from the CCl₄ model control group (P < 0.01, 0.05), especially the AST and ALT in ECCC group were reduced to (304.84 ± 150.84) and (305.96 ± 151.99) U/L, closer to those of normal control group.

The histological observation basically supported the results obtained from the serum enzyme assays as above mentioned. The liver sections from the control mice showed the normal lobular architecture and normal hepatic cells with a well-preserved cytoplasm and well-defined nucleus and nucleoli (Fig. 1). Histopathologic examination of livers challenged with CCl₄ showed centrilobular necrosis (N), ballooning degeneration, inflammatory infiltration, and fatty changes. The liver sections of mice pre-treated with ECC before CCl₄ challenged revealed that the extracts were able to prevent the development of histopathologic changes.

**Fig. 1  Histopathologic sections of liver by HE staining**

A: normal healthy rats, showing normal arrangements of cells in liver lobule  B: hepatocytes N and evident vacuolation (V) of hepatocytes in CCl₄-treated rats  C: liver sections of animals pre-treated with Bifendate Pills (0.40 g/kg) showing mild V of hepatocytes  D and E: animal pre-treated with ECCW and ECCBA (0.40 g/kg) showing mild hepatocytic N and mild V of hepatocytes  F and G: animal pretreated with ECCEA and ECCC (0.40 g/kg) showing marked improvement in histology over CCl₄ control group  H: liver sections of animals pretreated with ECCPE (0.40 g/kg) showing mild V of hepatocytes

**Effects of ECC on BCG + LPS-induced liver injury in mice**

The protective activities of ECC on biochemical markers, commonly used for the evaluation of acute hepatic injury, in BCG + LPS-intoxicated mice were obvious (Table 2). After single injection of LPS, the serum activities of AST and ALT enzymes in hepatotoxic model group (Group II) were significantly increased (P < 0.01) to (293.7 ± 22.96) and (81.82 ± 9.62) U/L, respectively, as compared with those of the normal control group [(115.97 ± 27.04) and (61.83 ± 26.44) U/L]. The elevated levels of these biochemical parameters clearly indicated the damage of hepatic cells. The treatment in the positive control group (Group III;
Table 2  Protective activities of ECC on BCG + LPS-induced elevation of AST, ALT, and liver coefficient in mice (x ± s, n = 10)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pretreatment dose / (g·kg⁻¹)</th>
<th>AST / (U·L⁻¹)</th>
<th>ALT / (U·L⁻¹)</th>
<th>Liver coefficient / %</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (normal control)</td>
<td>vehicle</td>
<td>115.97 ± 27.04</td>
<td>61.83 ± 26.44</td>
<td>5.29 ± 1.34</td>
</tr>
<tr>
<td>II (CCl₄ model control)</td>
<td>vehicle</td>
<td>293.70 ± 22.96</td>
<td>81.82 ± 9.62</td>
<td>5.49 ± 0.72</td>
</tr>
<tr>
<td>III (positive control)</td>
<td>0.40</td>
<td>241.08 ± 35.76</td>
<td>70.53 ± 7.80</td>
<td>5.19 ± 1.07</td>
</tr>
<tr>
<td>IV (CCl₄ + ECCW)</td>
<td>0.40</td>
<td>261.81 ± 25.04</td>
<td>81.54 ± 14.63</td>
<td>5.43 ± 0.93</td>
</tr>
<tr>
<td>V (CCl₄ + ECCBA)</td>
<td>0.40</td>
<td>246.11 ± 40.84</td>
<td>73.88 ± 9.40</td>
<td>5.54 ± 0.52</td>
</tr>
<tr>
<td>VI (CCl₄ + ECCEA)</td>
<td>0.40</td>
<td>259.86 ± 46.31</td>
<td>57.48 ± 10.34</td>
<td>5.22 ± 0.62</td>
</tr>
<tr>
<td>VII (CCl₄ + ECC)</td>
<td>0.40</td>
<td>240.73 ± 40.64</td>
<td>70.84 ± 12.73</td>
<td>5.46 ± 0.59</td>
</tr>
<tr>
<td>VIII (CCl₄ + ECCPE)</td>
<td>0.40</td>
<td>293.28 ± 74.22</td>
<td>82.00 ± 31.42</td>
<td>5.56 ± 0.92</td>
</tr>
</tbody>
</table>

**P < 0.01 vs normal control group; *P < 0.05  **P < 0.01 vs LPS model control group

0.40 g/kg) for 7 d significantly reduced the activities of serum AST and ALT (P < 0.01). Pre-treatment of animals with different ECC (Groups IV—VIII, 0.40 g/kg) for 10 d obviously reduced the activities of serum AST and ALT, as compared with the group of CCl₄-treated alone. The results of AST and ALT from Groups VI and VII were significantly different from the LPS model control group (P < 0.01, 0.05).

The histological observation basically supported the results obtained from serum enzyme assays. The liver sections from the control mice showed normal lobular architecture and normal hepatic cells with a well-preserved cytoplasm and well-defined nucleus and nucleoli (Fig. 2). The histopathological examination of livers challenged with LPS showed centrilobular N, ballooning degeneration, inflammatory infiltration, and fatty changes. The liver sections of mice pretreated with the ECC before LPS challenge revealed a prevention of the development of histopathological changes.

Discussion

The fruit of *C. colocynthis* (Cucurbitaceae) is well known for its medicinal properties. The literature rarely mentions if seeds are present in the preparations involving ground fruit/pulp. Our study investigated the in vivo hepatoprotective activities of this plant using the fruit containing seeds, which contain a large number of cucurbitacins, such as dihydrocucurbitacin E, cucurbitacins E, dihydro-epi-iso-cucurbitacin D, dihydroiso-cucurbitacin B-25-acetate, and cucurbitacin E-2-O-β-D-glucopyranoside (Miao et al., 2012). Cucurbitacins are a group of plant-derived tetracyclic triterpenoids originally found in the plant of Cucurbitaceae. Plants containing cucurbitacins have been known for their antipyretic, analgesic, anti-inflammatory, antimicrobial, antitumor (Lee et al., 2011; Nordmann, 1991), and liver
protective activities (Nordmann, 1991; Peters, Farias, and Ribeiro do Valle, 1997; Bartalis and Halaweish, 2011) in folk medicine. This indicated the liver protective effect may be dependent on the cucurbitacins of *C. colocynthis*. Further research is needed to confirm the hypothesis.

CCl₄ is a potent chemical hepatotoxin, which is frequently used as a chemical inducer of experimental tissue damages (Gurpreet et al., 2011; Yang et al., 2011; Mohamed et al., 2011). Transient tissue disorders after the administration of CCl₄ are believed to be induced by trichloromethyl radical (CCl₃). This free radical induces an adverse reaction by forming other free radicals after its administration in the early stage between intracellular uptake and transformation into storage types. The obtained results in the present study confirmed that CCl₄ challenge caused hepatocellular damage, as clearly indicated by the marked elevation of serum enzymes (AST and ALT) activities, relating to non-toxicated mice.

It is commonly recognized that the immune factors, such as autoimmune stimuli and virus or parasite infection, are the predominant reasons of hepatic damage especially under hepatitis. The commonly used model of liver injury, which is often induced by chemicals, may not accurately reflect the clinical situation. BCG and subsequent LPS injection provoked the hepatic injury in mice (Ferluga, 1981), which has been considered as a useful experimental model for immunological liver injury (Zou et al., 2006). Therefore, in this study, a BCG + LPS-induced liver injury was used to investigate the hepatoprotective activities of different kinds of ECC in mice. Considering the well-accepted fact that drugs reducing the levels of those serum marker enzymes exert the hepatoprotective activity, our study strongly suggests in vivo potential of ECC to prevent the chemically-induced hepatotoxicity, indicating that the ECCEA and ECCC are the most effective fractions.

In both cases, the results of AST and ALT from treatment groups (Groups VI—VIII) were significantly different from the CCl₄ or BCG + LPS model control group ($P < 0.01$, 0.05). The only imperfect fact is the large standard deviation in all pre-treatment groups, which may indicate the persistence of inter-individual variability in drug response among live organisms.

References


