THE EFFECT OF EURYCOMA LONGIFOLIA J ACK (TONGKAT ALI) ON CARBON TETRACHLORIDE-INDUCED LIVER DAMAGE IN RATS

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We attempted to investigate possible hepatoprotective effect of Eurycoma longifolia Jack (ELJ) using carbon tetrachloride (CCl₄)-induced acute hepatotoxicity model in rats. Hepatotoxicity was induced by oral administration of 4.0 mg/kg of CCl₄ (single dose) in corn oil (1:1) to one experimental group of 5 rats. In three other similar groups, doses (300, 750 and 1500 mg/kg respectively) of ELJ were given one day before and one hr after the administration of 4.0 mg/kg CCl₄ and then once daily for three consecutive days (D₁, D₂, D₃). Three other groups of 5 rats each serving as controls were administered with either distilled water, corn oil or ELJ (750 mg/kg) respectively. Rats were sacrificed on D₁ (corn oil and CCl₄ treated groups) or on day 4 (D₄) [distilled water, ELJ and CCl₄ with graded doses of ELJ treated groups]. Samples of blood or liver tissue were taken for biochemical (serum) and histopathological examinations to assess hepatoprotection of ELJ against CCl₄-induced hepatotoxicity. In the low (300 mg/kg) and medium (750 mg/kg) doses of ELJ-treated groups, CCl₄ was found to induce moderate inflammation, fatty acid change and necrosis of hepatocytes while in the high (1500 mg/kg) dose of ELJ, CCl₄ induced severe inflammation, fatty acid change and necrosis of hepatocytes. Biochemical measurements of alanine aminotransferase (ALT) and alkaline phosphatase (ALP) showed a moderate and insignificant reduction of serum levels in the low dose ELJ group but a more significant reduction in the medium and high dose ELJ groups when compared with the CCl₄-only group. The increase in serum total bilirubin (Tb) caused by CCl₄ was non-significantly reduced by all the doses of ELJ. Animals treated with CCl₄ alone and in groups treated with both CCl₄ and graded doses of ELJ had a reduction in body weight, food and water intake. In 750 mg/kg ELJ treated group, no such reduction in body weight, food and water intake was observed. This observation suggest that ELJ administered alone did not cause any toxic effect to the liver but in combination with CCl₄ appeared to synergise the CCl₄-induced hepatotoxicity which increases as the dose of ELJ is increased. The anorexic, hypodipsic and reduced body weight evident in the CCl₄-only and CCl₄ ELJ treated groups but not in animals treated with ELJ alone group, suggests that ELJ alone does not induce anorexia, hypodipsia or loss of weight. In conclusion, the results of our study suggest that ELJ is not hepatotoxic when given alone and appeared to have some degree of protective effects in rats against CCl₄-induced hepatotoxicity.

Keywords: Eurycoma longifolia Jack, CCl₄-induced liver damage, Rats

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INTRODUCTION

_Eurycoma longifolia_ Jack (ELJ, Tongkat Ali), Family Simarubaceae is a medicinal plant having widespread use among South East Asian nations. The root extract of ELJ is widely used in the form of beverage to improve sexual (aphrodisiac) functions (Ang, Ikeda and Gan 2001; Ang, Lee and Kiyoshi 2003), in addition to treatment of fever (satayavivad et al. 1998), jaundice and parasites. However, its hepatoprotective potential has not been studied. Using carbon tetrachloride (CCL4)-induced hepatotoxicity model in rats, we attempted to determine this probable effect. An attraction to this study is the observed similarity in chemical structure of ELJ to some herbs such as Carnosol, a medicinal herb with hepatoprotective activity (Sotelo-Felix, Martinez-Fong and Muriel De La Torre 2002). Furthermore, ELJ is popularly consumed by people of South East Asian nations and a hepatoprotective activity if any, would be an added advantage to its use.

METHODS

Plant Material

The 100% dried water extract of ELJ was purchased from MKI (M) Sdn. Bhd. (469700-V) Kuala Lumpur, Malaysia.

Animals and Management

Forty male Sprague Dawley rats (weighing between 100 and 196 g each) and mice pellet were obtained from Universiti Putra Malaysia (UPM) and maintained under standard conditions (temperature 24°C, light/darkness cycles of 12 h) with free access to food and water. All handling and management procedures were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Kulliyyah of Medicine, International Islamic University Malaysia (IIUM).

Chemicals

CCL4 (MW 153.82, purity 99.5% and density 1.592-1.598) was purchased from Avondale Chemicals Ltd., Banbury, Oxon, UK. Kits for enzyme [alanine aminotransferase (ALT) and aspartate amino transferase (AST)] measurement were purchased from Diagnostic System (Germany), while kits for alkaline phosphatase (ALP) and total bilirubin (Tbil) were obtained from Bayer (Germany). Other chemicals such as alcohol and formalin were of analytical grade and were purchased through IIUM and ether was obtained from Sigma Life Science (Germany).

Experimental Procedures

Treatment groups

A randomised block design was used to allocate the rats into groups. Rats were divided into 7 groups of 5 each for the main experiment and the 8th group of 5 rats was used for LD50 studies as follows:

Group I: Orally administered with distilled water (as control) in equivalent volume to the ELJ alone doses for three days (D₁, D₂, D₃) at the same time that the ELJ doses were administered to animals in groups II, V, VI and VII. On day four (D₄) all rats were sacrificed.

Group II: Orally administered with ELJ 750 mg/kg body weight (BW) (as control) on D₁, D₂, D₃. On D₄ all rats were sacrificed.

Group III: Orally administered with corn oil (as control) on D₂ in equivalent volume to CCl₄ administered group; at the same time that the CCl₄ dose was administered to animals in groups IV–VII. On D₃ all rats were sacrificed.

Group IV: Orally administered CCl₄ (2.5 mL/kg BW) diluted in corn oil (1:1) as a single dose on D₂. On D₃ all rats were sacrificed.

Groups V, VI and VII: Orally administered with water extract of ELJ (300, 750 and 1500 mg/kg BW) respectively, on D₁ and D₂ of experiment, and then 1 hr after the second dose, rats were given CCl₄ once orally at a dose of 2.5 mL/kg BW diluted in corn oil (1:1). On D₃ the rats were given same doses of ELJ only. On D₄ all rats were sacrificed.

Groups VIII: Administered with oral dose of ELJ (3000 mg/kg BW) one rat per day for five days.

Animal dosing

All animals were dosed orally by gavages using syringe and metal ball-ended needle specially designed for oral dosing.

Acute toxicity (LD₅₀) determination for ELJ

Oral acute lethal dose studies were carried out (to determine the safety profile) using the revised Up and Down (UPD) procedure according to recommendation (EPA 1998; OECD 1998). Since ELJ is dietary and most extracts from dietary plants tend to have high LD₅₀ and because herbal extracts with LD₅₀ above 3000 mg/kg/oral are considered safe, we used this dose as our limit dose. The limit test procedure was then performed as follows: a limit dose of 3000 mg/kg of the aqueous crude extract of ELJ extract was orally administered to the first rat (by gavage) and the animal was observed for mortality and/or clinical signs of toxicity, before, during and every 15 min after dosing for the first hr, then hourly for 3 hr, and then periodically for 72 hr. The remaining 4 rats were dosed in sequence at 48 hr interval. The LD₅₀ of ELJ was predicted to be above 3000 mg/kg if 3 or more rats survived.

Blood sampling and processing

Blood was taken through intra-cardiac puncture in the anesthetised rats using syringe with needle (23 G) and collected in test tubes and was kept at room temperature for 30 min for clot formation. Thereafter blood was centrifuged at 3000 rpm for 10 min and serum was collected and kept in refrigerator at −20°C until needed.
Measurement of liver enzymes

The enzymes ALT and AST levels were measured following the procedures outlined by the manufacturer Diagnostic System (Germany). The ALP and Tbil levels were also measured using biochemical analysis machine, model Express Plus 10591-5097 (USA).

Assay procedure

ALT and AST measurement were carried out using spectrophotometer; wavelength 340 nm, optical path 1 cm, temperature 37°C; measurement carried out against air. 100 µL of sample (serum) was pipetted into a test tube and was followed by the addition of 1000 µL of Reagent 1. The 2 were mixed and incubated for 5 min and thereafter, 250 µL of Reagent 2 was added and mixed. A sample of the mixture was then poured into a cuvette and put in the spectrophotometer for measurement of the enzyme activity (Hou, Qin and Ren 2010).

Determination of ALT and AST level

From the absorbance readings, ΔA/min was calculated and multiplied by the corresponding factor 2143 in order to obtain AST and ALT activities [U/I] using the following formula: ΔA/min x factor = AST, ALT activities [U/I].

Liver tissue sampling and processing

Immediately after sacrificing the animals, whole liver from each animal was obtained, washed in normal saline, weighed and its colour observed. The liver tissue specimens obtained from each liver was sliced into small sizes (3 mm in thickness) then put in tissue paper in small cassette plates fixed in formaldehyde (10%) and embedded in paraffin. The histological samples were cut into 5 µm sections and stained with haematoxylin and eosin (H and E stain).

The liver specimens were evaluated for presence or absence of fatty change, necrosis and inflammation according to lesion scores based on area of tissue involved. The lesion scores were determined using a 3-point scoring system on tissue sections as follows; 1=mild lesion (<33 % of tissue area involved), 2=moderate lesion (>33–<66%) and 3=severe lesion (>66%) (Chen et al. 2010).

Statistical Analysis

All values are expressed as the mean±SEM obtained from five animals. For statistical analysis, one way analysis of variance (ANOVA) and post hoc comparison of groups were employed using the SPSS statistical package 13. In all cases, a significant difference was considered when p value <0.05.
RESULTS

Determination of the Safety Profile of ELJ through LD₅₀ Study

None of the 5 rats died nor showed any sign of toxicity at the limit dose of 3000 mg/kg orally in the first 48 hr and no evidence of toxicity was noted during 4 days of observation. LD₅₀ of ELJ in rats was therefore taken as above 3000 mg/kg orally.

CCl₄-induced Changes in Biochemical Parameters and the Effect of ELJ Pretreatment

Figures 1–4 show the activities of ALP, Tbil, ALT and AST following CCl₄-induced rat hepatotoxicity and the effect of pretreatment of graded doses of ELJ. In addition to causing hepatotoxicity, CCl₄ also caused general loss of weight. The animals were inactive and did not eat or drink as compared with the normal rats (Fig. 5). The CCl₄-treated group remained inactive until D₂ when they were sacrificed.

Alkaline phosphatase (ALP)

CCl₄ significantly (p<0.001) increased ALP level with a value of 745.61±46.6 μmol/L when compared with a corn oil with the value 356.2±35 μmol/L. The low dose of ELJ (300 mg/kg BW) did not affect CCl₄-induced increase in ALP level. However, the medium dose (750 mg/kg BW) and high dose (1500 mg/kg BW) of ELJ significantly (p<0.001) and dependently caused a reduction of CCl₄-induced increase in ALP level with the value 495.8±9.8 μmol/L and 286.4±26 μmol/L respectively (Fig. 1).

Total bilirubin (Tbil)

CCl₄ did not significantly increase the Tbil level when compared to the corn oil group. The CCl₄-induced increase in Tbil was not significantly affected by any of ELJ treatment doses (Fig. 2).

Alanine amino transferase (ALT)

CCl₄ induced a significant increase (p<0.001) in ALT level (218.41±4.2 μmol/L) compared with corn oil with the value 22.52±1.32 (μmol/L). The low dose of ELJ did not significantly affect CCl₄-induced increase in ALT level. However, both the medium dose (750 mg/kg BW) with the value 171.8±4.7 μmol/L (p<0.001) and high dose (1500 mg/kg BW) with the value 189.9±4 μmol/L of ELJ significantly (p<0.001) prevented CCl₄-induced increase in serum ALT level (Fig. 3).

Aspartate amino transferase (AST)

CCl₄ induced a significant (p<0.001) increase in serum AST level (293.46±10.5 μmol/L) when compared with corn oil (control) (63.22±3.6 μmol/L). The low and high doses of ELJ did not significantly affect CCl₄ induced increase in AST level. However medium dose of ELJ with a value of 223±2 significantly (p<0.02) prevented CCl₄ induced increase in AST level when compared with CCl₄ treated group with the value 293.46±10.7 (μmol/L) (Fig. 4).
Notes: ** $p<0.001$ versus corn oil  
++ $p<0.001$ versus CCl₄

Fig. 1: The effect of various doses of ELJ on CCl₄-induced increase ALP.

Fig. 2: The effect of various doses of ELJ on CCl₄-induced increase Tbil.
Notes: ** p<0.001 versus corn oil
+ p<0.02 versus CCl4

Fig. 3: The effect of various doses of ELJ on CCl4-induced increase ALT.

Notes: ** p<0.001 versus corn oil
+ p<0.02 versus CCl4

Fig. 4: The effect of various doses of ELJ on CCl4-induced increase AST.

Body Weight Food and Water Intake

Animals treated with either distilled water, ELJ or corn oil (alone) showed slight increase in body weight over the 4-day treatment period. Animals in these groups also consumed rat chow and water normally without hindrance, and the amount of water and food intake were not significantly different between those groups. However animals administrated

with CCl₄ only and with various doses of ELJ failed to eat and drink normally when compared with the distilled water, ELJ or corn oil (alone) treated groups (Fig. 5 and 6). Also, animals in these groups appeared to show a decrease in body weight measured over the treatment period (Table 1).

**Fig. 5:** The effect of distilled water, corn oil, CCl₄ and various doses of ELJ on food intake.

**Histological Examination of the Liver Tissue**

The histopathological appearances of liver tissue shown in Figures 7 to 10 are represented by (a) x10 and (b) x40 magnification. In Figure 11 however, both (a) and (b) are x 40 magnification for low and high dose ELJ treatment, respectively. Sections from the liver in both control (distilled water) group [Fig. 7(a) and 7(b)] and ELJ (750 mg/kg) treated group [Fig. 8(a) and 8(b)] showed normal histological appearance. The liver of all rats administered with CCl₄ alone (4 g/kg) showed severe degree of fatty change, moderate inflammation and cirrhosis [Fig. 9(a) and 9(b)]. Rats treated with low and medium doses (300 and 750 mg/kg) [Fig. 10(a) and 10(b)], as well as high dose (1500 mg/kg BW) of ELJ...
when given before and after CCl₄ (4 mg/kg BW), showed moderate and severe degree of fatty acid changes, necrosis, inflammation and early cirrhosis in hepatic lobules and portal areas [Fig. 11(a) and 11(b)], respectively.

Fig. 6: The effect of distilled water, corn oil, CCl₄ and various doses of ELJ on water intake.

<table>
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<tr>
<th>Treatment groups</th>
<th>Rats</th>
<th>BW on D₁</th>
<th>BW on D₂</th>
<th>BW on D₃</th>
<th>BW on D₄</th>
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<tr>
<td>H₂O</td>
<td>5</td>
<td>148.4±12.6</td>
<td>150.8±12.5</td>
<td>155±13.3</td>
<td>159.4±12.9</td>
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<tr>
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<td>5</td>
<td>151.2±10</td>
<td>154±9.8</td>
<td>157.8±9.6</td>
<td>159±8.8</td>
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<tr>
<td>Corn oil only</td>
<td>5</td>
<td>142.6±3.6</td>
<td>144.6±3.6</td>
<td>148.2±3.3</td>
<td>+</td>
</tr>
<tr>
<td>CCl₄ only</td>
<td>5</td>
<td>131.8±7.43</td>
<td>134±7.3</td>
<td>129.2±7.1*</td>
<td>+</td>
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<tr>
<td>ELJ (300 mg) + CCl₄</td>
<td>5</td>
<td>152±10.3</td>
<td>146.8±10.1</td>
<td>140±10.5</td>
<td>134.4±9.5**</td>
</tr>
<tr>
<td>ELJ (750 mg) + CCl₄</td>
<td>5</td>
<td>140±12.6</td>
<td>136±12.6</td>
<td>126.6±11.9</td>
<td>120.8±12.4**</td>
</tr>
<tr>
<td>ELJ (1500 mg) + CCl₄</td>
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<td>142±9.5</td>
<td>138±9.3</td>
<td>130.4±9.1</td>
<td>124.4±9.2**</td>
</tr>
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</table>

Notes: *animals sacrificed  
* p<0.05 versus corn oil  
**p<0.05 versus ELJ (750mg/kg)

DISCUSSION

It is well established that hepatotoxicity by CCl₄ is due to enzymatic activation to release CCl₃ radical in free state, which in turn disrupts the structure and function of lipid and protein macromolecule in the membrane of the cell organelles (Mujumdar, Upadhye and Pradhan 1998). CCl₄-induced hepatotoxicity is being used as a model especially in assessing potential therapeutic agents including herbal medicines for possible protective effects (Rajesh and Latha 2004; Porchezhian and Ansari 2005). Hepatotoxic effect of CCl₄ has been achieved in our study as demonstrated by significant increases in serum ALP, ALT and AST as well as to the observed increases in enzyme levels, histopathological examination of rat’s liver following CCl₄-induced injury revealed severe inflammation, fatty change and necrosis. The administration of low and medium doses of ELJ one day before and one hr before CCl₄ as well as daily for three days after administration of CCl₄ moderately reversed the histopathological changes in acute liver injury caused by CCl₄. In the high dose ELJ-treated group, the degree of necrosis, fatty acid change and inflammatory process was evidently much less, and with a correspondingly lower levels of ALP and ALT. This observation suggests that ELJ administered alone did not affect or cause any toxic effect to the liver but in combination with CCl₄ appeared to reduce the CCl₄-induced hepatotoxicity. The lower levels of these liver enzymes (ALP and ALT) at higher doses of ELJ group (Fig. 3 and 4), and the apparent reduction of Tbil by the ELJ doses (Fig. 2), further attest the protective role to the hepatocytes such that fewer liver enzymes are released from the hepatocytes. Evidence of hepatoprotective effect of ELJ has previously been demonstrated in a related study in which the methanol-water extract (500 mg/kg) dosed consecutively for seven days suppressed the liver enzymes ALT and AST.
Fig. 7: Liver section of a rat treated with distilled water (control) showing a normal histological appearance. H and E staining; magnification (a) ×10 and (b) ×40.

Fig. 8: Liver section of a rat treated with ELJ alone (750 mg/kg) showing a normal histological appearance. H and E staining; magnification (a) ×10 and (b) ×40.
Fig. 9: Liver section of a rat treated with CCl₄ alone showing severe degree of fatty change, mild necrosis and moderate inflammation. H and E staining; magnification (a) × 10 and (b) × 40.

Fig. 10: Liver section of a rat treated with (a) low dose (300 mg/kg BW) and (b) medium dose (750 mg/kg BW), of ELJ after CCl₄ respectively showing moderate degree of fatty changes, necrosis and inflammation. H and E staining; magnification (a) × 10 and (b) × 40.
Fig. 11: Liver section of a rat treated with high dose (1500 mg/kg BW) of ELJ after CCl₄ showing severe degree of fatty changes, necrosis and inflammation. H and E staining; magnification, (a) × 40 and b× 40.

(Ruqiah et al. 2007). Our study has therefore, provided further evidence of hepatoprotection by ELJ. In the present study, animals treated with CCl₄ were seen to experience anorexia as evidenced by the failure of these animals to eat or drink leading to a visible reduction in their body weight. Anorexia, hypodypsia and reduced body weight is also evident in all the CCl₄ and ELJ-pretreated groups but not in animals treated with ELJ alone suggesting that ELJ alone does not induce anorexia, hypodypsia or loss of weight. The anorexic, hypodypsic and body weight reducing effects of CCl₄ have been well documented (Uemitsu and Nakayoshi 1984; Okamoto and Okabe, 2000; Okamoto et al. 2001).

CONCLUSION

The results of our study suggest that ELJ was not hepatotoxic and appeared to have some hepatoprotective effect against CCl₄-induced hepatotoxicity especially at higher doses. In contrast to CCl₄ ELJ did not cause anorexia, hypodypsia or weight loss. Further study is needed to further elucidate the extent and possible mechanism of the interaction of ELJ with CCl₄.
REFERENCES


