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THE EFFECT OF *EURYCOMA LONGIFOLIA* JACK (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 (CCl_4)-induced acute hepatotoxicity model in rats. Hepatotoxicity was induced by oral administration of 4.0 mg/kg of CCl_4 (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 CCl_4 and then once daily for three consecutive days (D_1, D_2, D_3). 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 D_3 (corn oil and CCl_4 treated groups) or on day 4 (D_4) [distilled water, ELJ and CCl_4 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 CCl_4-induced hepatotoxicity. In the low (300 mg/kg) and medium (750 mg/kg) doses of ELJ-treated groups, CCl_4 was found to induce moderate inflammation, fatty acid change and necrosis of hepatocytes while in the high (1500 mg/kg) dose of ELJ, CCl_4 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 CCl_4-only group. The increase in serum total bilirubin (Tbil) caused by CCl_4 was non-significantly reduced by all the doses of ELJ. Animals treated with CCl_4 alone and in groups treated with both CCl_4 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 CCl_4, appeared to synergise the CCl_4-induced hepatotoxicity which increases as the dose of ELJ is increased. The anorexic, hypodypsic and reduced body weight evident in the CCl_4-only and CCl_4 ELJ treated groups but not in animals treated with ELJ alone group, suggests that ELJ alone does not induce anorexia, hypodypsia 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 CCl_4-induced hepatotoxicity.

Keywords: *Eurycoma longifolia Jack, CCl_4-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 (CCl_4)-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

CCl_4 (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 8^{th} group of 5 rats was used for LD_{50} studies as follows:

Group I: Orally administered with distilled water (as control) in equivalent volume to the *ELJ* alone doses for three days (D_1, D_2, D_3) at the same time that the *ELJ* doses were administered to animals in groups II, V, VI and VII. On day four (D_4) all rats were sacrificed.

Group II: Orally administered with *ELJ* 750 mg/kg body weight (BW) (as control) on D_1, D_2, D_3. On D_4 all rats were sacrificed.

Group III: Orally administered with corn oil (as control) on D_2 in equivalent volume to CCl_4 administered group; at the same time that the CCl_4 dose was administered to animals in groups IV–VII. On D_3 all rats were sacrificed.

Group IV: Orally administered CCl_4 (2.5 mL/kg BW) diluted in corn oil (1:1) as a single dose on D_2. On D_3 all rats were sacrificed.

Group V, VI and VII: Orally administered with water extract of *ELJ* (300, 750 and 1500 mg/kg BW) respectively, on D_I and D_2 of experiment, and then 1 hr after the second dose, rats were given CCl_4 once orally at a dose of 2.5 mL/kg BW diluted in corn oil (1:1). On D_3 the rats were given same doses of *ELJ* only. On D_4 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 (LD_{50}) 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 LD_{50} and because herbal extracts with LD_{50} 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 LD_{50} 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. l00 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 LD_{50} 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. LD_{50} of *ELJ* in rats was therefore taken as above 3000 mg/kg orally.

CCl_4-induced Changes in Biochemical Parameters and the Effect of *ELJ* Pretreatment

Figures 1–4 show the activities of ALP, Tbil, ALT and AST following CCl_4-induced rat hepatotoxicity and the effect of pretreatment of graded doses of *ELJ*. In addition to causing hepatotoxicity, CCl_4 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 CCl_4-treated group remained inactive until D_2 when they were sacrificed.

Alkaline phosphatase (ALP)

CCl_4, 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 CCl_4-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 CCl_4-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)

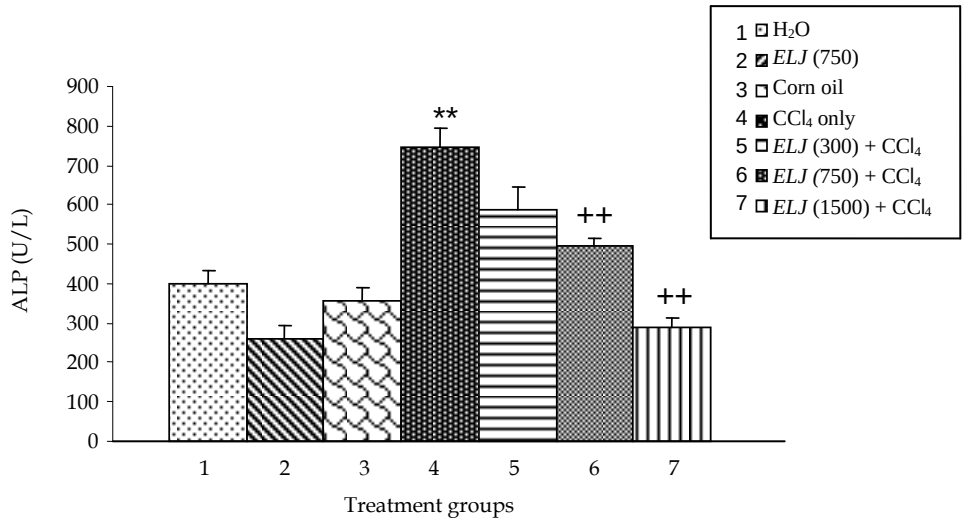
CCl_4 did not significantly increase the TBil level when compared to the corn oil group. The CCl_4-induced increase in TBil was not significantly affected by any of *ELJ* treatment doses (Fig. 2).

Alanine amino transferase (ALT)

CCl_4 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 CCl_4-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 CCl_4-induced increase in serum ALT level (Fig. 3).

Aspartate amino transferase (AST)

CCl_4 induced a significant (p<0.001) increase in serum AST level (293.46±10.5 µmo/L) when compared with corn oil (control) (63.22+3.6 µmo/L). The low and high doses of *ELJ* did not significantly affect CCl_4 induced increase in AST level. However medium dose of *ELJ* with a value of 223±2 significantly (p<0.02) prevented CCl_4 induced increase in AST level when compared with CCl_4 treated group with the value 293.46±10.7 (µmol/L) (Fig. 4).



Notes: ** p<0.001 versus corn oil
++p<0.001 versus CCl_4

Fig. 1: The effect of various doses of *ELJ* on CCl_4-induced increase ALP.

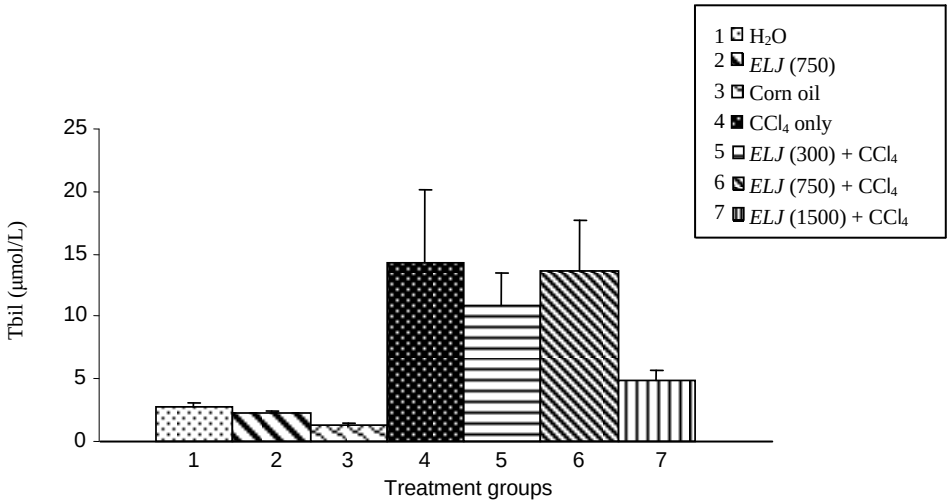
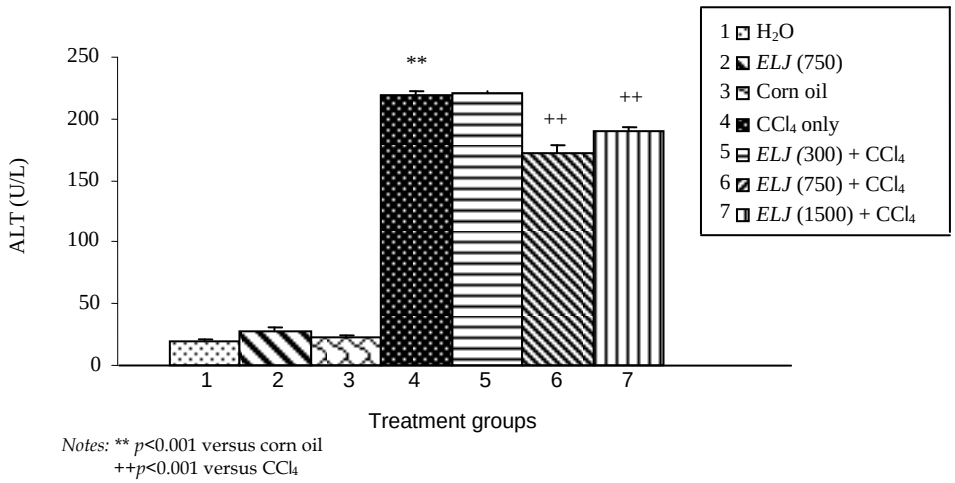
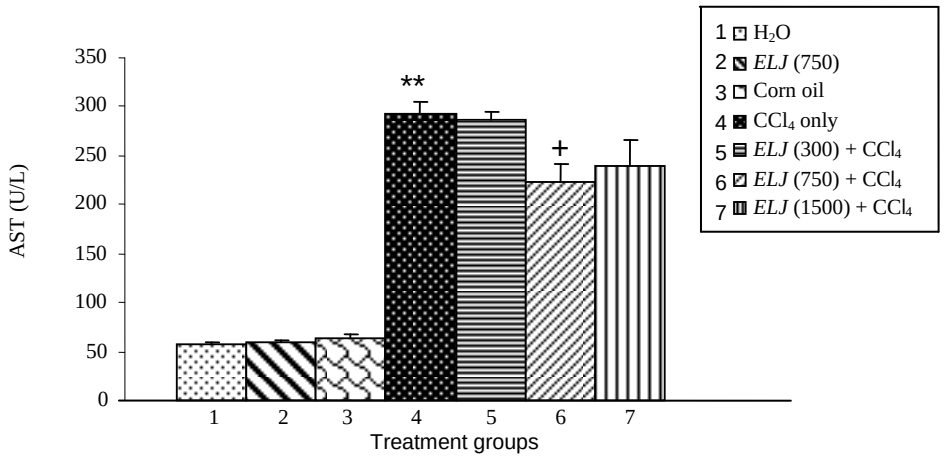


Fig. 2: The effect of various doses of *ELJ* on CCl_4-induced increase Tbil.



Notes: ** p<0.001 versus corn oil
++p<0.001 versus CCl_4

Fig. 3: The effect of various doses of *ELJ* on CCl_4-induced increase ALT.



Notes: ** p<0.001 versus corn oil
+p<0.02 versus CCl_4

Fig. 4: The effect of various doses *of ELJ* on CCl_4-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 CCl_4 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).

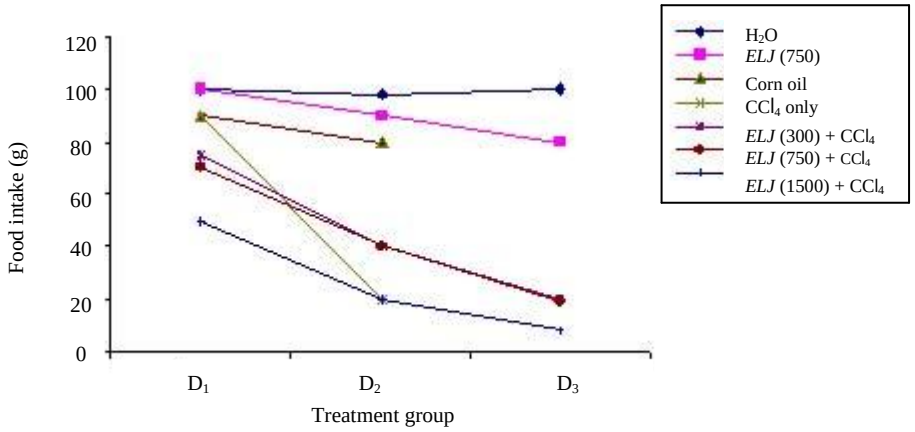
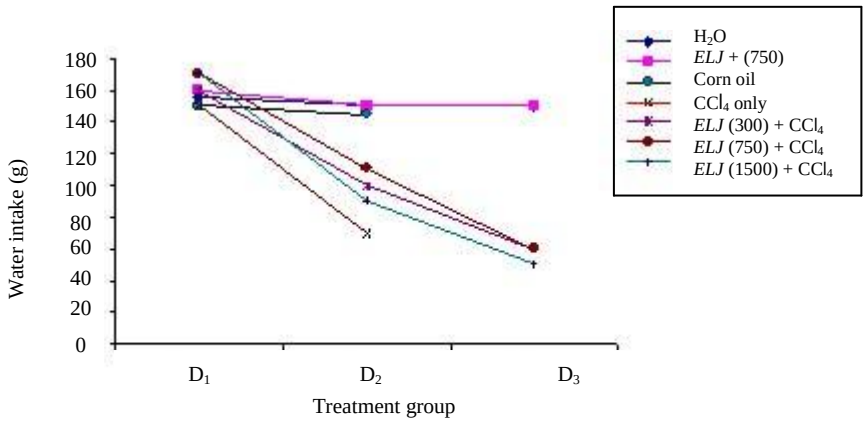


Fig. 5: The effect of distilled water, corn oil, CCl_4 and various doses of *ELJ* on food intake.



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 CCl_4 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 CCl_4, (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, CCl_4 and various doses of *ELJ* on water intake.

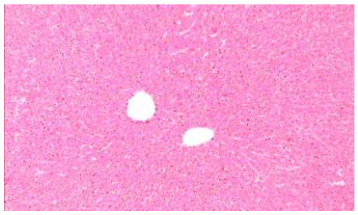
Table 1: BW changes in different experimental groups over the treatment period.

Treatment groups	Rats	BW on D_1	BW on D_2	BW on D_3	BW on D_4
H_2O	5	148.4±12.6	150.8±12.5	155±13.3	159.4±12.9
ELJ (750 mg/kg) only	5	151.2±10	154±9.8	157.8±9.6	159.6±8.8
Corn oil only	5	142.6±3.6	144.6±3.6	148.2±3.3	+
CCl_4 only	5	131.8±7.43	134±7.3	129.2±7.1*	+
ELJ (300 mg) + CCl_4	5	152±10.3	146.8±10.1	140±10.5	134.4±9.5**
ELJ (750 mg) + CCl_4	5	140±12.6	136±12.6	126.6±11.9	120.8±12.4**
ELJ (1500 mg) + CCl_4	5	142.2±9.5	138±9.3	130.4±9.1	124.4±9.2**

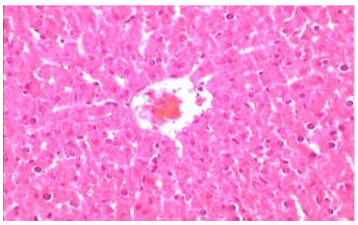
Notes: +animals sacrificed
* $p<0.05$ versus corn oil
**$p<0.05$ versus *ELJ* (750mg/kg)

DISCUSSION

It is well established that hepatotoxicity by CCl_4 is due to enzymatic activation to release CCl_3 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). CCl_4-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 CCl_4 has been achieved in our study as demonstrated by significant increases in serum ALP, ALT and AST as well as in Tbil. Besides to the observed increases in enzyme levels, histopathological examination of rat's liver following CCl_4-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 CCl_4 as well as daily for three days after administration of CCl_4, moderately reversed the histopathological changes in acute liver injury caused by CCl_4. 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 CCl_4, appeared to reduce the CCl_4-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

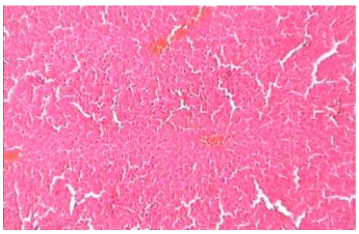


(a)



(b)

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.



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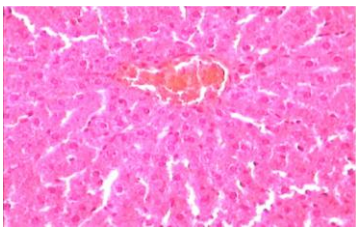
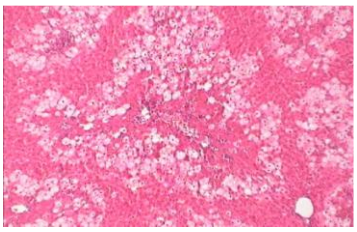


(b)

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.



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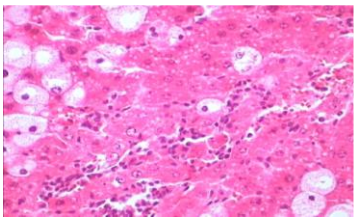
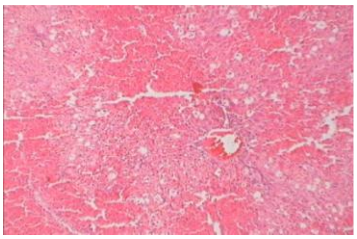


(b)

Fig. 9: Liver section of a rat treated with CCl_4 alone showing severe degree of fatty change, mild necrosis and moderate inflammation. H and E staining; magnification (a) × 10 and (b) × 40.



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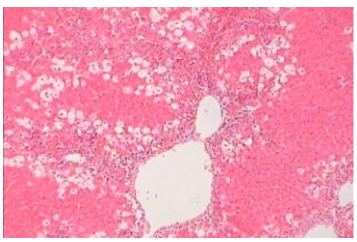
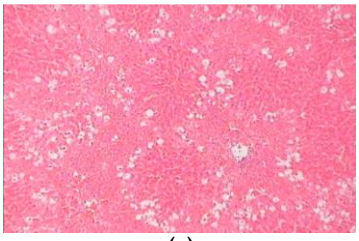


(b)

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 CCl_4 respectively showing moderate degree of fatty changes, necrosis and inflammation. H and E staining; magnification (a) × 10 and (b) × 40.



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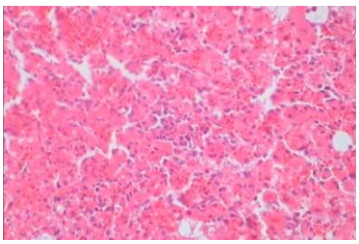

(b)

Fig. 11: Liver section of a rat treated with high dose (1500 mg/kg BW) of ELJ after CCl_4 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 CCl_4 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 CCl_4 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 CCl_4 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 CCl_4-induced hepatotoxicity especially at higher doses. In contrast to CCl_4, *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 CCl_4.

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