Toxicity of *Jatropha curcas* phorbol esters in mice

Cai-Yan Li [a], Rakshit K. Devappa [b], Jian-Xin Liu [a,*], Jian-Min Lv [c], H.P.S. Makkar [b,*], K. Becker [b]

[a] College of Animal Science, Zhejiang University, 310029 Hangzhou, PR China
[b] Institute for Animal Production in the Tropics and Subtropics, University of Hohenheim, 70593 Stuttgart, Germany
[c] Laboratory Animal Research Center, Zhejiang Chinese Medicine University, 310053 Hangzhou, PR China

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ABSTRACT

Phorbol esters are the main toxins in *Jatropha curcas* seed and oil. The aim of this study was to assess the acute toxicity of phorbol esters given by intragastric administration and to determine the LD_{50} for Swiss Hauschka mice. The LD_{50} and 95% confidence limits for male mice were 27.34 mg/kg body mass and 24.90–29.89 mg/kg body mass; and the LD_5 and LD_{95} were 18.87 and 39.62 mg/kg body mass, respectively. The regression equations between the probits of mortalities (Y) and the log of doses (D) was $Y = -9.67 + 10.21 \log(D)$. Histopathological studies on the organs from the dead mice showed: (1) no significant abnormal changes in the organs at the lowest dose (21.26 mg/kg body mass) studied, (2) prominent lesions mainly found in lung and kidney, with diffused haemorrhages in lung, and glomerular sclerosis and atrophy in kidney at doses $\geqslant$32.40 mg/kg body mass, and (3) multiple abruption of cardiac muscle fibres and anachromasis of cortical neurons at the highest dose of 36.00 mg/kg body mass. The results obtained would aid in developing safety measures for the Jatropha based biofuel industry and in exploiting the pharmaceutical and agricultural applications of phorbol esters.

1. Introduction

Jatropha curcas is a multipurpose bush or a small tree belonging to the family of *Euphorbiaceae*. It is a native of tropical America, but now thrives in many parts of the tropics and sub-tropics in Africa, Asia and southern America (Gübitz et al., 1999). The plant is well adapted to barren or drought affected areas and even survives in poor stony soils. *J. curcas* seed contains high amount of oil that can be converted into biodiesel of high quality upon transesterification. Apart from the oil, the seed cake or kernel meal leftover has gained tremendous interest for their utilization in feed formulations (Makkar et al., 1997). *J. curcas* kernel meal is rich in protein and the essential amino acid composition of the protein, except lysine, is comparable to that of soybean meal (Makkar et al., 1998; Devappa and Bhagya, 2008). However, the main toxins present in these by-products are phorbol esters, which prevent their utilization as feed ingredients (Makkar et al., 1997).

towards rabbit (100 μl), mice and rats (50 μl). The common symptoms of topical application were erythema, oedema, necrosis, diarrhoea, scaling and thickening of the skins (Gandhi et al., 1995). Feeding of *J. curcas* seeds, fruits or leaves caused toxicity depending on the dose and the animal model tested. Raw or defatted seeds when force-fed to fish, chicks, pigs, goat, mice and rats (caused severe toxicity symptoms before death (Liberalino et al., 1988; Chivandi et al., 2000; Gadir et al., 2003; Adam and Magzoub, 1975; Adam, 1974). Various organic and aqueous extracts also exhibited different toxic symptoms depending on dose, mode of administration and sensitivity of the animals being tested (Trebien et al., 1988; Mariz et al., 2006, 2008). For example, acetonitrile extract of *J. curcas* (seed or oil) when given to albino rats at an oral dose of 50 mg/kg body mass (single dose) produced mild toxicological, biochemical and histopathological changes (Abd-Elhamid, 2004). The methanol, petroleum ether and dichloromethane extracts of *J. curcas* fruit caused foetal resorption indicating pregnancy terminating effect in rats (Goonesekera et al., 1995). The irritant methanol fraction from *J. curcas* oil induced tumor promotion upon topical initiation by 7, 12-dimethylbenz(*a*)anthracene (DMBA) in mice, with 36% of the animals having skin tumors in 30 weeks (Horiuchi et al., 1988).

Hitherto, all the LD_{50} studies, toxicity studies or the histopathological studies conducted were using either *J. curcas* oil, seed, defatted seed cake or crude fractions from them. In the present study we purified phorbol esters from *J. curcas* oil, evaluated their acute toxicity when administered intragastrically and determined LD_{50} for laboratory mice.

2. Materials and methods

2.1. Materials

The *J. curcas* oil used in the study was from toxic genotypes from India. Corn oil was obtained from Sigma Chemical Company (Darmstadt, Germany). All other chemicals were of analytical grade.

2.2. Phorbol esters isolation and quantification

Phorbol esters were determined in quadruplicate according to Makkar et al. (2007), which was based on the method of Makkar et al. (1997). Briefly, 0.5 g of the sample was extracted four times with methanol. A suitable aliquot was loaded on a high-performance liquid chromatography (HPLC) reverse-phase C18 LiChrospher 100, 5 μm (250 × 4 mm I.D. from Merck (Darmstadt, Germany) column. The column was protected with a head column containing the same material. The separation was performed at room temperature (23 °C) and the flow rate was 1.3 ml/min using a gradient elution (Makkar et al., 2007). The four-phorbol esters peaks were detected at 280 nm and appeared between 25.5 and 30.5 min. For LD_{50} studies, the phorbol esters were carefully collected at the above retention times. All the collected fractions were in approximately 90% acetonitrile. These fractions were pooled and kept in a freezer (−20 °C). To avoid oxidation by water, the top acetonitrile layer from the frozen sample was separated and the acetonitrile was rotary evaporated at low vacuum to collect the colorless oily fraction. This fraction was redissolved in methanol and subjected to HPLC for checking the purity and concentration. The results were expressed as equivalent to phorbol-12-myristate 13-acetate.

2.3. Test substance

Phorbol esters were isolated from the *J. curcas* oil as above and diluted in high purity corn oil (Acros Organics Co., Geel, Belgium) for oral administration.

2.4. Animals and conditions

All the conditions used for animal housing and handling were approved by Animal Care and Use Committee at Zhejiang Province (China), and the experimental protocols used followed the Regulations for the Administration of Affairs Concerning Experimental Animals (The State Council of People's Republic of China, 1988).

A total of 70 male specific pathogen-free (SPF) grade Swiss Hauschka mice (21 days old) were obtained from Shanghai Laboratory Animal Center of China Academy of Science (Shanghai, China). The number of laboratory-animal-quality certification was SCXK (Hu) 2003-0003. The mice with an initial body mass of 17–18 g were kept in a large clean 27 × 17 × 13 cm polycarbonate cages (5 mice per cage) with bedding and fed on a laboratory diet (radiated by Co^{60}) for 3 days for acclimatization. After acclimatization, the mice were weighed and numbered.

The experiment was conducted in a barrier system with an experimental facility (License No: SYXK (ZHE) 2003-0003). The housing conditions were controlled automatically with a room temperature of 22 ± 1 °C; relative humidity 50–70%; lighting 150–200Lx, the sequence being 12 h dark and light cycle; noise <50 dB; air cleanliness degree 10,000 grade. During the experiment the mice had *ad libitum* access to food and sterile drinking water.

2.5. Test procedure

The test procedures were in accordance with the Regulatory Guide on the Techniques for Drug Research (The State Food and Drug Administration of People's Republic of China, 2005). A pretest was conducted to observe the range of toxicity so that the proper dose levels could be established for LD_{50} determination. Three dose levels (6, 12 and 18 mg/kg body mass) of phorbol esters were used for the pretesting. Based on the pretest results, six dosages (36.00, 32.40, 29.16, 26.24, 23.62 and 21.26 mg/kg body mass) were established with each group comprised of 10 mice using random block design (average body mass 18–20 g). The mice were kept fasting for 12 h before the phorbol esters doses were given by intragastric administration. The dosage of phorbol esters was given to mice as 0.2 ml/10 g body mass. The remaining 10 mice served as control and were given an equal volume (0.2 ml/10 g body mass) of corn oil. After the oil or phorbol ester administration, the mice in each group were fed as normal, and had free access to food and drinking water.

In the following 19 days, the clinical signs, change in body mass, and toxicity symptoms in mice were observed every day.

2.6. Histopathological studies

All deaths were recorded. Died mice and mice killed by cervical dislocation at the end of experiment were examined for gross and microscopical changes. The specimens of tissues from kidneys, liver, heart, spleen, brain and the lungs were taken for histopathological examination. The tissues were immediately rinsed with physiological saline, fixed overnight in 4% paraformaldehyde, and then dehydrated in a graded series of ethanol and embedded in paraffin for later slices and haematoxylin and eosin staining.

2.7. Calculations and statistical analysis

The LD_{50} determination was based on dose levels that increased by a geometrical progression. Six dose levels were ultimately required for establishing the LD_{50}. Calculations for LD_{50}, 95% confidence limits, LD_5 and LD_{95} were based on the Bliss method as explained in Zhou (1988). The Bliss was calculated by using the NDST Software Version 8.0 (Sun, 1998).

Table 1
Mortality of mice after intragastric administration.

Dose (mg/kg)	Dead	After administration (days)														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15–19
0 (Control)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
36.00	9	0	1	0	2	2	3	0	0	0	0	0	0	0	1	0
32.40	7	0	2	2	1	1	0	0	0	0	0	1	0	0	0	0
29.16	7	0	1	2	2	0	0	1	1	0	0	0	0	0	0	0
26.24	4	0	0	1	0	0	2	0	1	0	0	0	0	0	0	0
23.62	3	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0
21.26	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0

All the experimental analysis were statistically analyzed using the General Linear Model (GLM) Procedure of SAS by Duncan's new multiple range test to determine the significant differences in each concentration doses ($p < 0.05$).

3. Results

3.1. Mortality and live weight changes

The starting material had a phorbol ester concentration of 45.03 mg/g in corn oil. The death of mice occurred in a dose dependent manner (Table 1). Mice began to die on the 2nd day after administering the phorbol esters, and no more mice died during the extended 15–19 observation days. The time to death seems to depend on the administered dose and the individual susceptibility of mice to the phorbol esters. The higher the dose or less the resistance of an individual, the quicker the animal died.

Changes in body mass after administration of phorbol esters are shown in Table 2. For all doses, body mass of the phorbol esters administered mice decreased significantly after 3 days of the administration ($p < 0.05$). However, at the doses of 21.26–32.40 mg/kg, the body mass of mice those survived for 7 days after phorbol esters administration started reverting back to normal. At the highest dose of 36.00 mg/kg, the recovery of body weight oc-

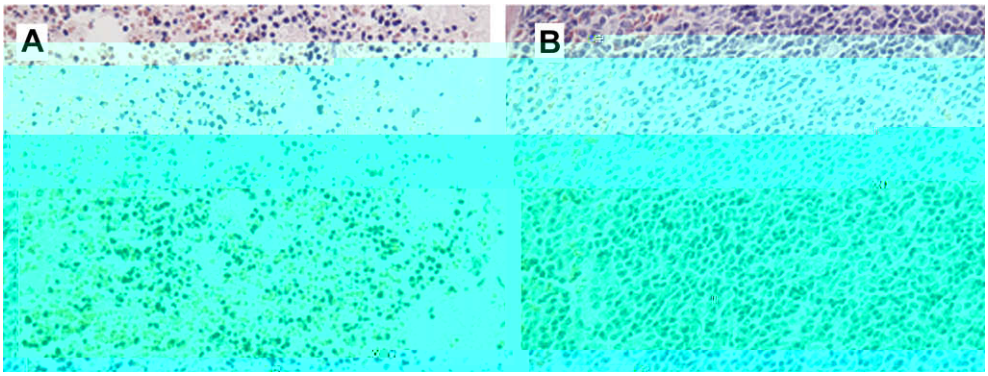

Fig. 2. Pathological changes of spleen observed at a dose of 36.00 mg/kg (A) Widely hyperemia and exudates (B) Normal spleen.

the gastro-intestinal tract haemorrhage may result in the black digesta in intestines. The stool findings showed that the digestive system of mice is sensitive to the presence of phorbol esters.

Histopathological studies of the organs showed normal cellular architecture at the lowest dose (21.26 mg/kg). At a dose of 23.61 mg/kg, no evidence of damage to heart, small intestine, brain and spleen was seen; however, sporadic infiltrated lymphocyte in the liver, seldom haemorrhage in lung and a few of glomerular sclerosis appeared. At a dose of 26.24 mg/kg, congestion of sinus hepaticus, haemorrhage of spleen and a few of glomerular atrophy were seen. At a dose of 29.31 mg/kg, congestion of the pulmonary alveolar capillaries was observed. When the dose increased to 32.40 mg/kg, much more pathological symptoms were seen in lung, with more mice showing congestion of the pulmonary alveolar capillaries and a few of mice exhibiting haemorrhage and burst of alveolus. At the highest dose of 36.00 mg/kg, multiple abruption of cardiac muscle fibres and anachromasis of cortical neurons appeared. Other histopathological changes included the frequent appearance of fatty vacuoles in the liver cells (Fig. 1), widely hyperemia and exudate in spleen (Fig. 2), diffuse haemorrhage and exudate in lung (Fig. 3), and glomerular sclerosis (Fig. 4). Overall, the prominent pathological symptoms were mainly observed in lung and kidney.

4. Discussion

Among the different methods available for LD_{50} determinations, the sensitivity of the Bliss procedure used in the present study is high, despite its complexity (Li et al., 1995).

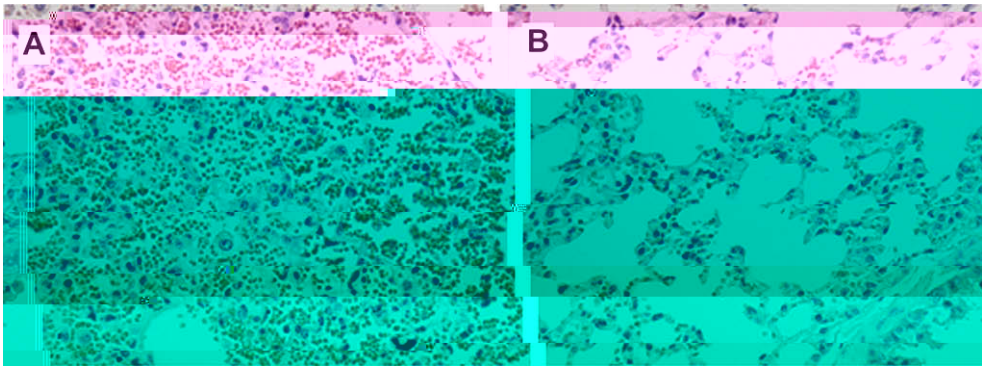

Fig. 3. Pathological changes of lung observed at a dose of 36.00 mg/kg (A) Widely diffuse haemorrhage and exudates (B) Normal lung.

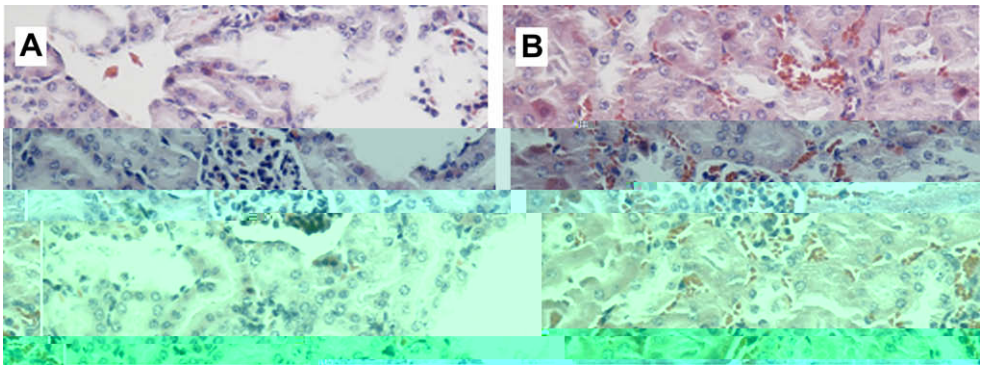

Fig. 4. Pathological changes of kidney observed at a dose of 36.00 mg/kg (A) Glomerular sclerosis (B) Normal kidney.

The LD_{50} study indicates that the purified phorbol esters isolated from the oil are highly toxic to mice and produce severe pathological symptoms. These results support the findings of Makkar et al. (1997, 1998) that phorbol esters are the main toxins in *J. curcas* oil and seeds. Phorbol esters are present in leaves, stems, flowers and roots of *J. curcas* (Makkar and Becker, 2009) and therefore the consumption of *J. curcas* in any form, oil, seeds, seed cake or extracts is toxic to animals, and elicits severe pathological symptoms. In the ruminants, force-feeding (drenching) studies using decorticated *J. curcas* seeds (kernels) caused acute toxicity with 100% mortality depending on the dose administered (Table 4). This highlights the importance of complete removal of phorbol esters from the Jatropha meal before using it in feed formulations. In many parts of the world, *J. curcas* is used as a live fence and the presence of phorbol esters in different parts of the plant and their toxicity is responsible for this use of the plant.

Taking average phorbol esters concentration, determined in our laboratory in the seeds of a number of toxic Indian variety of *J. curcas*, we back calculated the LD_{50} reported for oil by Gandhi et al. (1995) and for methanol extract from oil reported by Oluwole and Bolarinwa (1997). The calculated oral LD_{50} is 19.19 mg/kg body mass (analyzed average value of 3.5 mg/g taken for calculation) in rats and 49.87 mg/kg (intraperitoneal) in mice, respectively (analyzed average value of 1.98 mg/g taken for calculation). Rats fed with diet containing defatted whole seed Jatropha meal (10% protein replacement level) caused severe pathological symptoms and death occurred at a phorbol esters concentration of 47.31 mg/kg body mass (Rakshit et al., 2008).

The symptoms and toxicity of *J. curcas* depend on extract, dose, mode of administration and sensitivity of the animals being tested. For example, topical application of petroleum ether extract of *J. curcas*

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