Ecotoxicology and Environmental Safety 94 (2013) 172–178

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Occular and dermal toxicity of *Jatropha curcas* phorbol esters

Rakshit K. Devappa, Joy S. Roach, Harinder P.S. Makkar*, Klaus Becker

Institute for Animal Production in the Tropics and Subtropics (480b), University of Hohenheim, Stuttgart 70599, Germany

ARTICLE INFO

Article history:
Received 9 December 2012
Received in revised form
27 March 2013
Accepted 23 April 2013
Available online 21 May 2013

Keywords:
Dermal
In vitro
Jatropha curcas
Occular
Phorbol esters
Toxicity

ABSTRACT

Jatropha curcas seeds are a promising feedstock for biodiesel production. However, *Jatropha* seed oil and other plant parts are toxic due to the presence of phorbol esters (PEs). The ever-increasing cultivation of toxic genotype of *J. curcas* runs the risk of increased human exposure to *Jatropha* products. In the present study, effects of *J. curcas* oil (from both toxic and nontoxic genotypes), purified PEs-rich extract and purified PEs (factors C_1, C_2, $C_{3mixture}$, (C_4+C_5)) on reconstituted human epithelium (RHE) and human corneal epithelium (HCE) were evaluated *in vitro*. The PEs were purified from toxic *Jatropha* oil. In both RHE and HCE, the topical application of PEs containing samples produced severe cellular alterations such as marked oedema, presence of less viable cell layers, necrosis and/or partial tissue disintegration in epithelium and increased inflammatory response (interleukin-1α and prostaglandin E_2). When compared to toxic oil, histological alterations and inflammatory response were less evident ($P<0.05$) in nontoxic oil indicating the severity of toxicity was due to PEs. Conclusively, topical applications of *Jatropha* PEs are toxic towards RHE and HCE models, which represents dermal and occular toxicity respectively. Data obtained from this study would aid in the development of safety procedures for *Jatropha* biodiesel industries. It is advised to use protective gloves and glasses when handling PEs containing *Jatropha* products.

1. Introduction

Jatropha curcas is a promising feedstock for biodiesel production (Makkar and Becker, 2009). During the biodiesel production, many coproducts such as nitrogen rich seed cake, phytochemicals, and glycerol, among others could be generated (Makkar and Becker, 2009). However, the presence of toxic compounds (phorbol esters (PEs) and curcin) limits their utilisation. There are many reports describing the toxic effects of *Jatropha* seed, oil or extracts in higher animals (mice, rat, rabbit, sheep, among others), micro-organisms, insects and humans (Devappa et al., 2010a, Gandhi et al., 1995a; Adam and Magzoub, 1975; Ahmed and Adam, 1979). In humans/animals, the exposure towards *Jatropha* based products are merely accidental or force fed (Devappa et al., 2010a). In majority of the toxicity studies, oil soluble PEs were regarded as the active component. Generally, PEs (mg/g dry matter) are distributed in most of the plant parts such as roots (0.55), bark (outer brown skin) (0.39), bark (inner green skin) (3.08), wood (0.09), stems (0.78–0.99), leaves (1.83–2.75), buds (1.18–2.10), flowers (1.39–1.83) and kernels (2–6). However, they are not found in the latex (Makkar and Becker, 2009).

PEs are the most potent and toxic tigliane diterpenes commonly found in the genera *Anthosthema*, *Croton*, *Euphorbia*, *Ostodes*, *Jatropha*, *Sapium* and *Wikstroemia* (Xu et al., 2009). These tigliane diterpenes are diversely oxygenated and hydroxylated in various esterified forms. Most of the PEs found in plants differ in their hydroxylation patterns and in the stereochemistry of their ring systems. Phorbol 12-myristate 13-acetate (PMA; synonym, 12-O-Tetradecanoylphorbol-13-acetate (TPA)) is the most studied PE within the Euphorbiaceae family. PMA exhibits diverse biological activities (both *in vitro* and *in vivo*), of which tumour promotion activity is the most investigated one. Phorbol esters promote tumour growth, following the exposure of an initiator chemical such as methylcholanthrene (MCA) or 7, 12-dimethylbenzanthracene (DMBA) (Goel et al., 2007). In principle, they act as analogues of diacyl glycerol (DAG), which is a secondary messenger in one of the main cellular signal transduction pathways. Generally, DAG is involved in the activation of enzyme protein kinase C (PKC) which further catalyses the phosphorylation of other proteins involved in signal transduction. The hyper activation of PKC by PEs (DAG analogue) targets multiple sites within a cell resulting in the uncontrolled cellular differentiation, which cumulatively results in the production of tumours (Kinzel et al. 1984; Goel et al., 2007). In addition to tumour promotion, PEs exhibit wide range of other biochemical and cellular effects; for example they alter cell morphology, serve as lymphocyte mitogen, induce platelet aggregation, elevate cyclic GMP levels, stimulate ornithine decarboxylase and exhibit anti-leukemic activities. The PEs are also found to be involved in the

* Corresponding author. Fax: +49 71145923702.
E-mail address: Harinder.Makkar@uni-hohenheim.de (H.P.S. Makkar).

0147-6513/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ecoenv.2013.04.021

modulation of inflammatory responses, inhibition of virus entry and effects on nociception (Goel et al., 2007). In addition, toxicity of PEs is structure dependent. Generally 'α' form of phorbol is inactive when compared to 'β' form (Goel et al., 2007).

During *Jatropha* biodiesel production, the PEs present in *Jatropha* oil are completely degraded. However, in early steps of the transesterification process PEs were still detected in the acid gums and in disposed water washings (Makkar et al., 2009). This suggests that one should be cautious when using acid gums in animal feed and also controlled disposal of water washings containing PEs is necessary to avoid ecotoxicity. As the scale of *Jatropha* production increases, so does the risk of eco-toxicological effects/exposure of *Jatropha* based products. This has raised concerns with respect to the consequences of occupational exposure of *Jatropha* oil (Gressel, 2008; Achten et al., 2008: Devappa et al., 2010b). In spite of high production and plausible applications of *Jatropha*, knowledge on the potential impacts of toxic *Jatropha* oil on human health and environment is still fragmentary.

In the present study, toxic genotype (toxic oil, PEs- rich extract and purified PEs) and nontoxic genotype (PEs free nontoxic oil) of *Jatropha* were evaluated for dermal and ocular toxicity using reconstructed human epithelium (RHE) and human corneal epithelium (HCE) models respectively. These *in vitro* test models (RHE and HCE) have been widely used for evaluating toxicity of chemicals. (Cotovio et al., 2007; Alépée et al., 2009; Kishore et al., 2009; Tornier et al., 2010; Goethem et al., 2006).

2. Materials and methods

2.1. Materials

J. curcas seeds (toxic Indian variety) were collected from wild trees (mature, approximate age 15 years) existing in places around Jaipur (geographical coordinates: 26°55′0″N, 75°49′0″E), Rajasthan, India. The phorbol 12-myristate-13-acetate (PMA) was obtained from Sigma Aldrich (St. Louis, MO, USA) and all other chemicals/solvents used were of analytical grade.

2.2. Extraction of Jatropha oil

J. curcas seeds (both toxic and nontoxic genotypes) were mechanically pressed using a screw press to obtain oil and seed cake. The oil was centrifuged (3150*g*, 20 min) to remove residues and the clear oil was collected and stored in a refrigerator (4 °C) until further use.

2.3. Quantification of PEs

The PEs were determined ($n=3$) as described by Makkar et al. 2007, based on the method of Makkar et al. (1997). Briefly, 0.5 g of phorbol ester containing samples were extracted with 1–1.5 mL solvent (99 percent methanol/1 percent THF) in a ball mill (MM200, Retsch GmbH & Co., Haan, Germany; 30 1/s) for 5 min. The supernatant was collected by centrifugation (12,500*g* for 3 min). Similarly, the residue was re-extracted (3 ×) and centrifuged to collect supernatant. The supernatant from four extractions was pooled together and concentrated using pressurised air to get oily fraction. The oily fraction was re-dissolved in methanol (1 mL). A suitable aliquot was loaded into a high-performance liquid chromatography (HPLC) fixed with a reverse-phase C_{18} LiChrospher 100, 5 mm (250 × 4 mm^2 i.d.), from Merck (Darmstadt, Germany) column and separation was carried out at 23 °C. The flow rate was 1.3 mL/min using a gradient elution. The four phorbol ester peaks appeared between 25.5 and 30.5 min and were detected at 280 nm. The concentration was expressed equivalent to both *Jatropha* factor C_1 and PMA (Makkar et al., 2007; Roach et al., 2012). The sensitivity (limit of quantification) of HPLC method for factor C_1 was 5.8 ng and for PMA was 200 ng.

2.4. Crude phorbol ester extraction

Jatropha oil used for the isolation of PEs contains 0.11 mg/g of PEs equivalent to *Jatropha* factor C_1 (or 4.6 mg/g of PEs, equivalent to PMA). *Jatropha* oil was mixed with methanol (1:2, w/v) in a capped container (60 °C for 15 min) using a magnetic stirrer (300 rpm). Thereafter, the mixture was centrifuged at 3,150*g* for 5 min to get upper methanolic and lower oily layers. Both the layers were separated. The oily layer was re-extracted three more times with the fresh solvent in a ratio of 1:1.5, 1:1 and 1:1 (w/v). The methanolic layers were pooled together and rotoevaporated (65 °C, 300 mbar) to get oily PEs enriched fraction (PEEF) (Devappa et al., 2010b).

2.5. Purification of PEs

In brief, the PEEF (see Section 2.4) was subjected to column chromatography (Silica G-60) to get a concentrated PEs-rich extract (*Jatropha* factors C_1–C_5) (Devappa et al., 2011; Roach et al., 2012). The concentrated PEs-rich extract was fractionated by semi-preparative HPLC to obtain individual PEs, which were purified on a Sephadex LH-20 column. The purified PEs (factors C_1 and C_2) were confirmed by 1D (^1H, ^{13}C) and 2D (HSQC, COSY, TOCSY, and HMBC) NMR and the data corresponded to data published by Haas et al. (2002). Whereas, factor C_3 and factors (C_4+C_5) were obtained as a mixture. Consequently, the concentration of *Jatropha* factors used in this study was expressed as *Jatropha* factor C_1 equivalents. The concentrated PEs-rich extract was also used in the study. PEs were stored in ethanol until further analysis at −80 °C.

2.6. Effect of phorbol esters on reconstructed human epidermis (RHE) and reconstituted human corneal epithelium (HCE)

The skin epithelium and corneal epithelium are the primary point of contact for a toxic material (Huhtala et al., 2008). Therefore, we have chosen the RHE and HCE models, which mimic the human skin and corneal epithelium respectively to test the toxicity of the PEs. In addition, release of inflammatory substances such as interleukins and prostaglandins was also evaluated.

2.6.1. Effect of phorbol esters on reconstructed human epidermis (RHE)

The RHE model closely resembles human epidermis *in vivo* (Doucet et al., 1998). The RHE cultures were purchased from SkinEthic Laboratories (Nice, France). In brief, the fully differentiated RHE was cultured using primary keratinocytes isolated from human foreskin. The culture was carried out on an inert microporous polycarbonate filter (0.5 cm^2) at the air–liquid interface for 17 days. The culture was maintained in chemically defined growth culture medium MCDB 153 (5 mg/mL insulin, 1.5 mM calcium chloride, 25 mg/mL gentamycin, and 1 ng/mL epidermal growth factor (EGF) (Rosdy and Clauss, 1990). The fully differentiated and stratified epidermis model obtained on day-17 consisted of main basal, supra basal, spinous and granular layers and a functional stratum corneum (Fartasch and Ponec, 1994; Kandárová et al., 2006a, 2006b). On day 17, tissues were transported from the firm to our laboratory on a nutritive agarose plate enclosed in an aluminium bag. The maintenance medium and growth medium was supplied by SkinEthic Laboratories along with RHE models. Upon arrival, tissues were transferred to another 6-well plates consisting of 1 mL growth culture medium. The plates consisting RHE tissues were incubated (37 °C, 5 percent CO_2 and 95 percent relative humidity) until test substance application. On day 19, the RHE tissues were transferred into another 24 well plate containing 300 μL pre-warmed maintenance culture medium. All the experimental procedure was carried out in sterile conditions. The experiment involves topical application of test materials on to the surface of epidermis and subsequent assessment of their effects on histology and inflammatory substances. The test substances (test material, negative and positive controls) were topically applied on RHE tissue and the RHE was exposed for 42 min at room temperature (22 °C). After 42 min, the RHE tissue was rinsed with Dulbecco's Phosphate-Buffered Saline (D-PBS), mechanically dried and transferred into fresh medium and then incubated (37 °C, 5 percent CO_2, 95 percent humidified atmosphere) for 42 additional hours.

The following test articles (10 μL) were applied ($n=2$) topically on RHE tissues (i.e. 20 μL/cm²): toxic *Jatropha* oil, nontoxic *Jatropha* oil, purified PEs (factor C_1, C_2, $C_{3mixture}$, (C_4+C_5)) and PEs-rich extract (factor C_1 to C_5 in the same proportion as they exist in the oil), 70 percent ethanol, 5 percent sodium dodecyl sulphate (5 percent SDS; positive control), phosphate buffered saline (PBS; negative control) and dimethylsulphoxide (DMSO; vehicle). The application of DMSO (10 μL) acted as blank. The aforementioned PEs containing test articles were dissolved homogeneously in DMSO and its (DMSO) concentration did not exceed 0.1 percent in the final application (10 μL). To ensure even spreading of test samples, a nylon mesh (8 mm diameter) was deposited onto the RHE tissues before application.

2.6.2. Effect of phorbol esters on reconstituted human corneal epithelium (HCE)

The HCE model was purchased from SkinEthic Laboratories (Nice, France). The model consists of immortalised human corneal epithelial cells cultured in a chemically defined medium and seeded on a synthetic membrane at the air–liquid interface. The tissue structure obtained was a multilayered epithelium (0.5 cm^2) resembling *in vivo* epithelium (Cotovio et al., 2007) representing about 5–7 cell layers. The HCE (age day 5–0.5 cm^2) was transported to our laboratory in a similar manner to the RHE. The inserts containing the HCE were transported in a multi well plate filled with an agarose-nutrient solution in which they were embedded. The maintenance medium and growth medium were shipped by the SkinEthic Laboratories® along with the HCE models. Upon arrival HCE was transferred to a new maintenance medium, 1 mL/well in 6 well plates and incubated at 37 °C, 5 percent CO_2 in a humidified incubator. The medium was renewed 24 h later. The