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Screening for toxic phorbol esters in jerky pet treat products using LC–MS

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ABSTRACT

Since 2007, the U.S. FDA's Center for Veterinary Medicine (CVM) has been investigating reports of pets becoming ill after consuming jerky pet treats. Jerky used in pet treats contains glycerin, which can be made from vegetable oil or as a byproduct of biodiesel production. Because some biodiesel is produced using oil from *Jatropha curcas*, a plant that contains toxic compounds including phorbol esters, CVM developed a liquid chromatography-mass spectrometry (LC–MS) screening method to evaluate investigational jerky samples for the presence of these toxins. Results indicated that the samples analyzed with the new method did not contain *Jatropha* toxins at or above the lowest concentration tested.

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1. Introduction

Since 2007, FDA's Center for Veterinary Medicine (CVM) has received numerous reports of pet illnesses following the consumption of jerky pet treats [1]. Reports involve treats from multiple manufacturers and include a range of brands and flavors; these treats typically contain chicken or duck breast meat, glycerin, and spices as their main ingredients. Efforts by numerous laboratories to determine the underlying cause of the treat-associated illnesses have been unsuccessful to date and have focused on screening jerky treats for various contaminants and toxic agents. Investigations have included microbiological, compositional, and chemical toxicological testing as well as studies to determine whether irradiation of jerky treats leads to the formation of potentially toxic compounds. Particular attention has been given to glycerin as a potential source of toxicants.

Jerky pet treats are generally made from poultry (chicken, duck, turkey, etc.) breast meat that has been soaked in glycerin and dried in an oven. Glycerin used in jerky treats may be produced from vegetable oil or as a byproduct of biodiesel manufacturing. One source of biodiesel fuel is oil from the seeds of *Jatropha curcas*, a toxic, drought-hardy plant prevalent in Latin American, Asian, and African countries [2]. If ingested, these plants can cause severe gastrointestinal irritation, dehydration, and death in humans and animals [3–5].

The toxicity of *J. curcas* is ascribed mainly to a toxic protein, curcin, and a group of diterpene esters termed phorbol esters, which are present in relatively high concentrations in the seeds of some *J. curcas* varieties [2]. While heat denatures curcin and renders it nontoxic, it does not affect phorbol esters [6]. Six *J. curcus* phorbol esters, named *Jatropha* factors (JFs) C1–C6, have been isolated and characterized previously (Fig. 1A-F) [2,7]. All six compounds have the same molecular formula and are intramolecular diesters of the diterpene, 12-deoxy-16-hydroxyphorbol (Fig. 1G). Upon mechanical extraction of oil from *Jatropha* seeds, the majority of JFs (~70%) remain in the oil fraction [8]. There is a concern, therefore, that these compounds are a potential cause of toxicity in jerky treats made using contaminated glycerin.

We developed an LC–MS method to determine if *Jatropha* toxins were present in jerky pet treats. Because standards of JFs were

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not commercially available, we used phorbol 12-myristate 13-acetate (PMA, Fig. 1H), a phorbol ester analogue, for preliminary method development work. The method was based on an initial solvent extraction followed by a clean-up using hydrophilic lipophilic polymeric solid phase extraction cartridges. The esters were chromatographically resolved and analyzed by mass spectrometry. The method yielded satisfactory recoveries of the JFs, and was suitable for the analysis of investigational samples. We used this new method to analyze several investigational jerky pet treat samples.

2. Materials and methods

2.1. Materials

HPLC grade methanol and acetonitrile were purchased from Burdick & Jackson (Muskegon, MI, USA). A Milli-Q Reference Ultrapure Water System (Millipore Corp., Billerica, MA, USA) was used to obtain deionized water. Formic acid (95%) and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Food-grade glycerin (99.7%) was purchased from US Pharmacopeia (Rockville, MD, USA). Oasis HLB SPE cartridges were purchased from Waters Corp. (Milford, MA, USA). Potassium phosphate buffer (0.3 M, pH 7) was prepared using analytical grade potassium dihydrogen phosphate and potassium monohydrogen phosphate purchased from Fisher Scientific (Fair Lawn, NJ, USA). Whatman 60 A, 70–230 mesh silica gel and silica gel 60 F254 2.5 × 7.5 cm were used for flash chromatography and thin layer chromatography, respectively.

J. curcas oil (25 mL) was obtained from F. Beckford, University of Florida, IFAS Extension through the CVM's Veterinary Laboratory Investigation & Response Network (Vet-LIRN). Vet-LIRN also provided investigational samples obtained from consumer cases and retail markets. Samples were stored at −15 °C on receipt. Pet jerky treat samples to be used as controls were prepared in our laboratory using store-bought fresh chicken breast.

2.2. Extraction of jatropha factors (JFs) from J. curcas oil

We extracted JFs from oil (25 mL) of *J. curcas* using previously described methods [8]. The *Jatropha* oil was extracted with methanol (25 mL) using a magnetic stirrer (300 rpm) at 60 °C for 5 min. The resulting mixture was gravity separated. After three extractions, the methanol layers were combined and evaporated under nitrogen and freeze dried to obtain ∼3 g of residue. The residue was dissolved in dichlomethane (DCM), loaded onto a silica gel column, and eluted with 1, 3, and 5% methanol in DCM. The fractions were monitored by thin layer chromatography under short wavelength UV. The JFs eluted with 3% methanol in DCM, which was evaporated to yield a crude standard enriched with JFs (∼16 μg).

2.3. Preparation of control jerky treat samples

Control jerky treat samples were made using chicken breast tenderloins bought in a grocery store. The tenderloins were soaked in food-grade glycerin for 20 min before drying. Tenderloins were placed on the dehydrator rack for 30 min (Sedona Dehydrator; Tribest Co., Anaheim, CA, USA) after soaking to allow excess glycerin to drip onto a paper towel placed under the rack. Samples were then dried at 68 °C for 48 h, left on the rack for 2 h to cool, and placed in labeled whirl pak bags. Prepared samples were stored at room temperature.

2.4. Sample extraction procedure

A schematic presentation of the sample preparation is given in Fig. 2. A jerky treat sample (50 g) was homogenized using a Robot Coupe blender (Robot Coupe USA Inc., Ridgeland, MS, USA) for 5–10 min until it was ground to a fine powder-like consistency. The ground sample was stored at −15 °C until analysis.

The ground sample was thawed and weighed (0.5 g) into a 15 mL polypropylene centrifuge tube. Potassium phosphate buffer (0.3 M, 0.5 mL) and methanol (1.5 mL) were added and the sample was

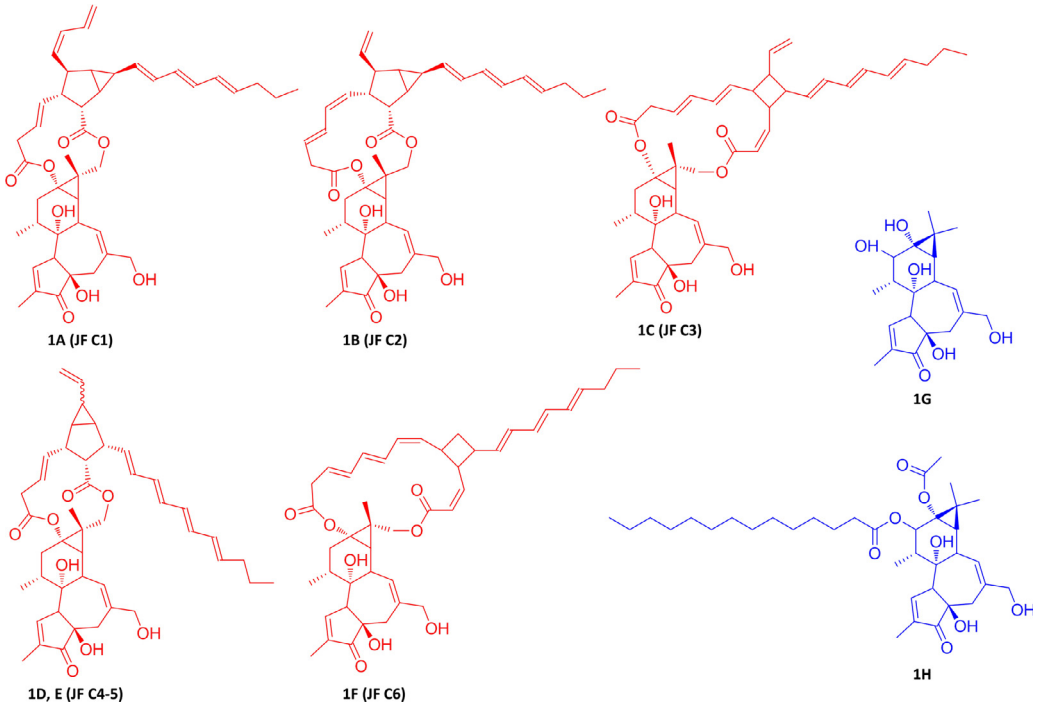

Fig. 1. Structures of *Jatropha* factors C1–C6 (1A–1F), 12-deoxy-16-hydroxyphorbol (1G) and phorbol 12-myristate 13-acetate (1H).