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# One year oral Toxicity of D-004, a lipid extract from *Roystonea regia* fruits, in Sprague Dawley rats

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ABSTRACT

D-004, a lipid extract of royal palm (*Roystonea regia*) fruits that contains a reproducible mixture of fatty acids, has been shown to prevent testosterone and phenylephrine-induced prostate hyperplasia in rodents. This study investigated the long-term oral toxicity of D-004 in rats. Rats from both sexes were randomized into four groups (20 rats sex/group): a control and three treated with D-004 (800, 1500 or 2000 mg/kg/day, respectively). At study completion, rats were sacrificed under anaesthesia. Determinations of blood biochemical and haematological parameters and organ weight were done. Also, necropsy and histopathological studies were performed. Four of 160 rats died before study completion. No clinical signs of toxicity were observed throughout the study. Food and water consumption, bodyweight, blood biochemical and haematological parameters, organ weight ratios and histopathological findings were similar in control and treated groups. The histological lesions found in treated animals are commonly present in this specie and strain according to literature and our historical data. In conclusion, long-term (12 months) oral treatment of rats with D-004 (800–2000 mg/kg/day) did not show evidences of D-004-related toxicity under our conditions. The highest dose tested (2000 mg/kg) was a no-observed adverse effect level in this study.



## 1. Introduction

Benign prostatic hyperplasia (BPH), a highly prevalent disease among older men, is a non-malignant prostate enlargement frequently accompanied of disturbing lower urinary tract symptoms (LUTS) (Connolly and Fitzpatrick, 2007; Lourenco et al., 2008; Nix and Carson, 2007; Sampson et al., 2008).

The pathogenesis of BPH, although not fully understood, involves hormonal and non-hormonal factors that occur in the ageing man (Connolly and Fitzpatrick, 2007; Lourenco et al., 2008; Roehrborn, 2008). The main hormonal factor that contributes to BPH is the increased conversion of testosterone (T) in dihydrotestosterone (DHT) by the prostate 5α-reductase enzyme, since DHT accumulation in the prostate promotes excessive prostatic cell growth (Carson and Rittmaster, 2003). In turn, the increased tone of prostate and bladder smooth muscle mediated through the α1-adrenoreceptors (ADR) is the main non-hormonal factor involved in BPH/LUTS (Schwinn and Roehrborn, 2008). Hence, 5α-reductase inhibitors and α1-ADR blockers are the cornerstone of the pharmacological therapy of BPH/LUTS (Jewett and Klotz, 2007). After months on therapy, prostate 5α-reductase inhibitors mainly reduce prostate enlargement and mildly ameliorate LUTS (Jewett and Klotz, 2007; Tarter and Vaughan, 2006); while α1-ADR blockers effectively reduce LUTS as soon as 2 weeks after starting the therapy (Jewett and Klotz, 2007; Lepor, 2006; Schwinn and Roehrborn, 2008; Yamada and Ito, 2011). The combined therapy with 5α-reductase inhibitors and α1-ADR blockers provides the benefits of both therapeutic classes (McVary, 2007; Roehrborn, 2008; Sandhu and Vaughan, 2005). Nevertheless, drug-related adverse effects (AE) are reported for all these drugs, so that 5α-reductase inhibitors cause impaired men sexual function (decreased libido, impotence, ejaculatory disorders) (Jewett and Klotz, 2007; Tarter and Vaughan, 2006), and α1-ADR blockers mainly produce orthostatic hypotension, dizziness, asthenia and ejaculatory disorders (Jewett and Klotz, 2007; Lepor, 2006; Miner et al., 2006).

D-004, a lipid extract obtained from the mature fruits of the royal palm by a process that includes a first alkaline hydrolysis and a further solvent (*n*/hexane) extraction, contains a mixture of free fatty acids (mainly oleic, lauric, palmitic and myristic acids) wherein oleic acid is the most abundant). Oral treatment with D-004 has been

*Abbreviations:* AchE, acetylcholinesterase; ADR, adrenoreceptors; AE, adverse effects; ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; BPH, benign prostatic hyperplasia; CENPALAB, National Centre for Laboratory Animals Production; DHT, dihydrotestosterone; LUTS, lower urinary tract symptoms; NOAEL, no-observed-adverse-effect level; PHE, phenylephrine; RBCs, red blood cells; T, testosterone; WBCs, white blood cells.

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shown to prevent T- and phenylephrine (PHE)-induced prostate hyperplasia in rodents (Arruzazabala et al., 2004; Carbajal et al., 2004, 2005; Noa et al., 2005). The main mechanisms whereby D-004 may produce these effects involve the inhibition of prostate 5α-reductase activity (Pérez et al., 2006) and the antagonism of α1-ADR-(Arruzazabala et al., 2005, 2006).

Studies of oral toxicity (single and repeat doses) of D-004 in rodents have not shown evidences of D-004-related toxicity when it was given to 2000 mg/kg/day for 90 and 60 days to rats and mice, respectively, so that 2000 mg/kg/day is a not observed adverse effect level (NOAEL) (Gámez et al., 2005b; Gutiérrez et al., 2008). Likewise, genotoxicity studies did not reveal D-004-related cytotoxic or genotoxic potential (Fernández et al., 2005; Gutiérrez et al., 2005).

Since therapy to treat BPH/LUTS will be administered long-term, the potential chronic toxicity of new substances addressed to such aim should be investigated. Keeping in mind this background, this study investigated the long-term (12 months) oral toxicity of D-004 in Sprague Dawley rats of both sexes.

## 2. Material and methods

### 2.1. Animals and housing conditions

Young adult Sprague Dawley rats of both sexes, 180–230 g, purchased at the Centre of Laboratory Animals Production (CENPALAB; Havana, Cuba), were adapted for 1 week to the experimental conditions of the entire experiment: temperature (25 ± 2 °C), humidity (50–70%) and 12-h light/dark cycles. Animals were housed in plastic shoeboxes and bedding (processed hardwood chips) was changed and sterilized in autoclave (Guide for the Care and Use of Laboratory Animals, 2011; Siglin and Baker, 2002).

Free access to tap water and food (CENPALAB rodent chow) was allowed during the study. At treatment completion, rats were fasted for 12 h prior to the sacrifice (Auletta, 2002; Barile, 2008; Gad, 2002; Wilson et al., 2001). Animals were handled in accordance to the Cuban Ethical Regulations for Animal Care and the Cuban Code of Good Laboratory Practices for Toxicological Studies (Regulatory Board for the Public Health Protection, 2004). The study protocol was approved by an independent ethical board.

### 2.2. Test substance

The batch used in the study, supplied by the Chemistry Department of the Centre of Natural Products (Havana, Cuba) and assessed with a validated gas chromatography method, had the following free fatty acid composition, caprylic 0.5%, capric 0.7%, lauric 23.1%, myristic 10.7%, palmitic 10.9%, palmitoleic 0.3%, stearic 2.4% and oleic 42.9%, purity being 91.5%. D-004 was suspended in Tween-65/water vehicle 1 h before dosing. The concentrations of these suspensions were adjusted weekly according to bodyweight gain.

Rats were randomised into four groups of 20 rats per sex: a vehicle (Tween-65/water) control group and three treated with D-004 (800, 1500 or 2000 mg/kg, respectively) for 12 months. Treatments (vehicle or D-004) were given once daily orally through/via gastric gavage (2 mL/kg) (6 days a week) (8:30–10:30 a.m.). Doses of 800 and 2000 mg/kg were the lowest and highest levels, respectively, considering: (a) the effective experimental doses of D-004 (200–800 mg/kg) (Arruzazabala et al., 2004; Carbajal et al., 2004; Noa et al., 2005), (b) the lack of D-004-related toxicity previously found (Gámez et al., 2005b; Gutiérrez et al., 2008) and (c) the acceptable upper limit dose for studies of oral chronic toxicity in rodents (1000 mg/kg) (OPPTS 870.4100 Chronic Toxicity, 1996).

### 2.3. Clinical observations haematology and clinical chemistry

Clinical observations were performed twice a day: in the morning (8:30–10:30 a.m.) and afternoon (4:00–5:00 p.m.). Appearance and overt behaviour of animals were recorded daily, so that any change in the skin and fur, eyes and mucous membranes, faeces and locomotor activity, and occurrence of salivation, lacrimation, tremors, convulsions, piloerection, stereotypes, evident masses or abscesses were registered.

Body weight and food consumption were determined at baseline, then weekly during the first 13 weeks and monthly thereafter (Barile, 2008; Siglin and Baker, 2002).

Moribund animals and those with relevant body weight reduction (⩾10%) should be euthanised during the study. At the end of the 12 months, survivors were isolated in individual cages, fasted for 12 h with free access to water, and then anesthetised under diethyl ether atmosphere and sacrificed by complete bleeding.

Blood was drawn from the abdominal aorta and samples were collected in non-heparinized and heparinized tubes for serum biochemical and haematological determinations, respectively, then placed at room temperature for 30 min and centrifuged at 3000 rpm for 10 min. Supernatant aliquots were taken to assess the following determinations: glucose, triglycerides, cholesterol, total protein, creatinine, urea and the enzyme activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, creatine kinase and acetylcholinesterase (AchE). Serum biochemical parameters were determined by using reagent kits (Randox; Crumlin Co., Antrim, UK), except blood acetylcholinesterase, determined in accordance to Voss and Sacsse (1970). Haematological parameters included haemoglobin, hematocrit, white blood cells (WBCs), red blood cells (RBCs) and platelet count, all assessed in the haematological SySMEX (model KX-21N; SySMEX Corporation, Kobe, Japan). All samples were processed within the same day of blood sampling.

### 2.4. Pathology

During the autopsy, the abdominal, thoracic and cranial cavities of all animals were examined and the liver, heart, kidneys, adrenal glands, testis, prostate, epididymis, seminal vesicles, levator ani bulbocavernosous muscles, bulbourethral and preputial glands, uterus, ovaries, spleen, lungs and thymus of all animals were weighed (Sartorius Universal Scale, Goettingen, Germany). The ratios of organ to body weight were determined and expressed as percent (Long et al., 1998).

Samples of the organs mentioned above and of lymph nodes (mandibular and mesenteric); bone white marrow; pituitary gland; thyroid with parathyroid; larynx/trachea; bronchi; salivary glands; tongue; oesophagus; stomach (glandular and non-glandular); small (duodenum, jejunum and ileum) and large intestine (caecum, colon and rectum); pancreas; penis; urinary bladder; vagina; skeletal muscle; skin and subcutis; eyes; Harderian glands; Zymbal gland; sciatic nerve; cerebrum; cerebellum and spinal cord were preserved in 10% buffered formaldehyde (Barile, 2008; Hall, 2007; Siglin and Baker, 2002).

Then, samples from all animals with macroscopic lesions, and from the control and highest dose groups, were taken and embedded in paraffin, sectioned with a rotary microtome (Leitz microtome, Wetzlar, Germany), stained with haematoxylin and eosin, and examined by light microscopy. An Olympus BH2 microscope (Olympus Optical Co., Ltd. Tokyo, Japan) was used for these observations.

### 2.5. Statistical analysis

Data were analysed following the recommendations for toxicological studies (Barile, 2008; Festing and Altman, 2002; Gad, 2001). Continuous data (bodyweight, blood parameters, food consumption and organ weight percentage) were analysed with analysis of variance (ANOVA) and categorical data (mortality, histological lesions) with the Fisher's Exact Probability test. An alpha value of 0.05 was *a priori* established. Tests were two tailed and the statistical analyses were performed independently by sex using the STATISTIC data analysis software (StatSoft, Inc. 2003; Tulsa, OK, USA, version 6. (www.statsoft.com).

## 3. Results

### 3.1. Mortality and clinical signs

Four of 160 rats (2.5%) died before study completion, one control male and three females (one control, two of D-004 2000 mg/kg). No significant differences in the mortality rate in control and treated groups were found. The death of one female treated with the highest dose of D-004 occurred immediately after dosing, when the animal suddenly had dyspnoea and progressive worsening of health status. Presence of D-004 emulsion into the respiratory conducts and the lungs was found during the necropsy; so that such death was attributed to wrong oral gavage procedure.

The other three premature deaths (one control male, one control female, one female of 2000 mg/kg) were euthanised because of evident weight loss and tumours in the legs that were histiocytomas (two controls), and vaginal prolapse and haemorrhage (the female of 2000 mg/kg) due to a polipoid vaginal tumour. The first sacrifice occurred after 8 months of treatment.

With the exception of the euthanised rats, the rest of the animals exhibited a good health during the study, so that no clinical signs of toxicity were observed.