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

A. Gutiérrez *, R. Gámez, M. Noa, R. Mas, D. Arencibia, B. Pardo, M. Valle, A. Oyarzábal, D. Curveco, H. García, E. Goicochea, N. Mendoza, S. Jiménez.

*Centre of Natural Products, National Centre for Scientific Research, P.O. Box 6990, 198 Ave. and 19 St. Cubanacán, Playa, Havana, Cuba*

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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.

* Corresponding author. Tel.: +53 271 4200; fax: +53 7 273 6837.
*E-mail address:* ariadne.gutierrez@cnic.edu.cu (A. Gutiérrez).

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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.

### 3.2. Effects on safety indicators

Bodyweight gain, food and water consumption were similar in treated and control groups in all the points of the study (data not shown for simplicity). Likewise, the values of bodyweight, haematological and blood biochemical parameters at study completion (Table 1–3) did not reveal significant differences between control and treated groups nor trends with the doses in any sex.

### 3.3. Pathology

Organ to bodyweight ratios did not show significant differences or trends with the doses (Table 4).

The most frequent non-neoplastic lesions were pituitary congestion and glomerulonephrosis. Several inflammatory lesions were found, all at low frequencies and without differences between treated and control groups (Table 5). Prostatitis, occurring like small foci of chronic inflammation in the stroma between the glands, was observed in five rats (one control, one of 1500 mg/kg and three of 2000 mg/kg).

In addition, tumours were found in three controls (one male, two females) and four treated (one male, three females) rats, including the lesions of the three euthanised rats referred above. Three histiocytomas were found in control animals (one benign in a male, two malignant in two females). One male treated with 1500 mg/kg had a benign pituitary adenoma, while three treated-females (one of each group) displayed an endometrial polyp (a rat of 800 mg/kg), one thymoma (a rat of 1500 mg/kg), and a leiomyoma (a rat of 2000 mg/kg. The rates of these lesions in control and treated groups were statistically similar (Table 6).

## 4. Discussion

This study demonstrates that long-term (1 year) oral administration of D-004, a lipid extract from *Roystonea regia* fruits that reduces T and PHE-induced prostate hyperplasia (Arruzazabala et al., 2004; Noa et al., 2005), did not produce treatment-related toxicity in rats, consistent with previous toxicological studies (Gámez et al., 2005b; Gutiérrez et al., 2008).

The mortality rate (4/160, 2.5%) was low for a 1 year study, as compared to others 1 year studies conducted in this species/strain (Alemán et al., 1994; Gámez et al., 2005a; Rodeiro et al., 1998), which supports that study conduct was good.

In accordance to daily observations, food consumption, weight gain, blood biochemical and haematological indicators, organ to bodyweight ratio and results of the histological study, no evidence of D-004 related toxicity was found, which agrees with previous results (Gámez et al., 2005b; Gutiérrez et al., 2008). Also, individual values of tested parameters were within normal limits reported for this species according to our historical data (Alemán et al., 1998; Gámez et al., 2005a; Johnson and Gad, 2007; Levine, 2002; Rodeiro et al., 1998). Keeping in mind that we administered D-004 at doses effective in rodents (Arruzazabala et al., 2004; Noa et al., 2005), the lack of toxicity here found should not been ascribed to an inadequate exposure to the substance. The present results were consistent with those of previous studies of the oral toxicity of D-004 that included assessment at earlier time points (Gámez et al., 2005b; Gutiérrez et al., 2008), so that the contribution of a mechanism of adaptation/recovery to the absence of toxicity may be ruled out.

The frequency of glomerulonephrosis, present in four control (two males, two females) (10%) and two treated rats of the 800 and 1500 mg/kg groups, respectively, is consistent with our previous data (Alemán et al., 1998) and with other reports (Giknis and Clifford, 2004; Greaves and Faccini, 1984), This renal disease, common in adult and aged rats; can appear as early as at 5 months (Johnson and Gad, 2007). The animals with this lesion present hyaline tubules and tubular epithelial cells with cytoplasmic changes (vacuoles and hyaline degeneration). The glomeruli show a wide variety of changes (focal or diffuse) and inflammatory cells in the interstitium. The pathogenesis of glomerulonephrosis, although not fully understood, seems to be associated to the dysfunction of the basal membrane (Greaves and Faccini, 1984).

Pituitary congestion, another frequent lesion, occurred in two control animals (5%), female rats and four treated with D-004 (3.3%), one male and two female rats treated with 800 mg/kg.

Keeping in mind the pharmacological action of D-004, we assessed carefully the reproductive organs, but we failed to find any sign of treatment-related toxicity on these targets and found that the relative organ weights of the testis and uterus of treated animals of both sexes were unaffected by the treatment. Four control males and only two D-004-treated exhibited testicular atrophy, a common lesion at the first year of life in this specie (Greaves and Faccini, 1984); with a rate of about 2% in young males (Peckham, 2002). This lesion affects the subcapsular tubules and appears at necropsy as small, watery, blue testis. Histologically, the affected tubules are small and show partial or complete lack of germ cells, leaving only Sertoli cells. This condition can result from congenital origin and obstructive lesions, and may be associated with relative increases of Sertoli cell numbers. It should be noted that some data support significant incidences of testicular atrophy in animals treated with high doses of substances attributable to non-specific stress rather than to specific target organ toxicity. In these cases, the animals also generally have reduced food consumption and body weight (Johnson and Gad, 2007). In our study, however, differences between treated and control groups were not significant.

Prostatitis, another genital lesion observed in five rats (one control, one of 1500 mg/kg, three of 2000 mg/kg) was not considered as treatment-related because not only the difference as compared to the controls was not significant, but because this lesion is very common in adult rats, as supported by Peckham (2002), (up to 14% in young rats).

All other histopathologic findings found in the study have exhibited similar frequencies in control and treated rats, without trends with the doses, have been found in our historical controls (Alemán et al., 1998; Gámez et al., 2005a), and have been reported as spontaneous for this strain/species (Peckham, 2002). All these arguments support that none of the other lesions are attributable to D-004 treatment.

Overall, seven rats (three euthanised, four sacrificed at study completion) exhibited some tumour. Three control rats (one male, two females) had histiocytomas: one benign, two malignant ones, respectively. This finding is not surprising, since these subcutaneous tissue tumours appear spontaneously in Sprague Dawley rats of this age (Giknis and Clifford, 2004; Peckham, 2002).

Four neoplastic lesions were found in treated rats. One male of the 1500 mg/kg group had a pituitary adenoma, a benign tumour that usually represents the most prevalent spontaneous neoplastic

**Table 1**
Effects of 1 year oral administration of D-004 on the rat bodyweight values (g) (mean ± SD).

| Group | Males | | Females | |
|---|---|---|---|---|
| | Baseline | 1 year | Baseline | 1 year |
| Control | 223.2 ± 9.0 | 613.5 ± 61.6 | 193.5 ± 9.3 | 373.7 ± 415 |
| *D004 (mg/kg/day)* | | | | |
| 800 | 223.2 ± 10.6 | 618.2 ± 76.3 | 193.5 ± 10.0 | 389.2 ± 62.1 |
| 1500 | 223.2 ± 9.9 | 606.7 ± 75.8 | 193.5 ± 7.9 | 369.8 ± 43.5 |
| 2000 | 223.2 ± 10.2 | 587.6 ± 79.1 | 193.5 ± 9.1 | 369.8 ± 42.3 |

*Note:* There were not significant differences between D-004-treated and control groups.

**Table 2**
Effects of 1 year oral administration of D-004 on the rat haematological parameters (mean ± SD).

| Treatment (mg/kg/day) | Red blood cell ($\times10^{-6}$/L) | Haemoglobin (g/100 mL) | Haematocrit (%) | Platelets ($\times10^{-3}$/L) |
|---|---|---|---|---|
| *Males* | | | | |
| Control | 7.53 ± 0.44 | 13.52 ± 0.76 | 42.95 ± 2.84 | 871.26 ± 239.64 |
| 800 | 7.33 ± 0.47 | 13.51 ± 0.62 | 42.26 ± 2.48 | 903.35 ± 150.36 |
| 1500 | 7.57 ± 0.67 | 13.62 ± 1.02 | 43.33 ± 3.59 | 921.30 ± 275.32 |
| 2000 | 7.56 ± 0.38 | 13.71 ± 0.71 | 43.30 ± 2.80 | 938.55 ± 192.18 |
| *Females* | | | | |
| Control | 6.43 ± 0.38 | 12.62 ± 0.65 | 38.33 ± 2.31 | 603.42 ± 201.41 |
| 800 | 6.47 ± 0.41 | 12.61 ± 0.60 | 38.27 ± 2.20 | 583.70 ± 255.80 |
| 1500 | 6.48 ± 0.36 | 12.57 ± 0.65 | 38.24 ± 2.01 | 641.55 ± 196.23 |
| 2000 | 6.46 ± 0.30 | 12.65 ± 0.58 | 38.30 ± 1.76 | 676.33 ± 296.73 |

| Treatment (mg/kg/day) | Differential leucocyte count (%) | | Total leucocyte count ($\times10^{-3}$/L) |
|---|---|---|---|
| | Lymphocytes | Neutrophils | |
| *Males* | | | |
| Control | 0.73 ± 0.10 | 0.27 ± 0.10 | 6.13 ± 2.01 |
| 800 | 0.75 ± 0.07 | 0.25 ± 0.07 | 6.41 ± 1.73 |
| 1500 | 0.74 ± 0.08 | 0.26 ± 0.07 | 6.38 ± 2.08 |
| 2000 | 0.75 ± 0.07 | 0.25 ± 0.07 | 6.14 ± 1.56 |
| *Females* | | | |
| Control | 0.70 ± 0.13 | 0.30 ± 0.13 | 3.17 ± 0.91 |
| 800 | 0.74 ± 0.11 | 0.26 ± 0.11 | 3.55 ± 1.17 |
| 1500 | 0.77 ± 0.09 | 0.23 ± 0.09 | 3.51 ± 1.11 |
| 2000 | 0.71 ± 0.10 | 0.29 ± 0.10 | 3.40 ± 0.98 |

*Note:* There were not significant differences between D-004-treated and control groups.

lesion in rats of this age (Johnson and Gad, 2007). Indeed, the frequency of this lesion was actually low as compared to the literature and our previous data, a fact without a conclusive explanation, but that does not limit the conclusions of this study focused to determine the oral chronic toxicity of D-004, not its potential carcinogenicity.

Two treated females displayed neoplastic lesions of the genital tract: one of 800 mg/kg exhibited a endometrial polyp, a benign tumour common in rats of this age (Giknis and Clifford, 2004; Greaves and Faccini, 1984; Peckham, 2002), and other of 2000 mg/kg had a benign vaginal leiomyoma, a tumour occasionally found in the rat female genital tract that may apparently extend into the vagina (Greaves and Faccini, 1984). Finally, the thymoma found in a female rat 1500 mg/kg represents another benign and spontaneous lesion of this species (Giknis and Clifford, 2004; Greaves and Faccini, 1984; Peckham, 2002).

This report summarises the results of the first long-term toxicity study of D-004, a new substance obtained from the fruits of the royal palm (*R.regia*). These fruits have been traditionally used in Cuba as pig, not human, food (Uhl and Dransfield, 1987; Zona, 1991). Then, safety data based on the extrapolation of traditional human exposure is practically absent.

Some indirect evidences, however, supported that the intake of D-004 should not suppose a relevant safety risk for humans. First, the composition of D-004 partially resembles that of the lipid extract of saw palmetto (*Serenoa repens*) fruits, widely used to treat BPH/LUTS, without producing relevant toxicity or adverse effects (Avins et al., 2008; Agbabiaka et al., 2009; Habib, 2009).

Second, since animals and humans have consumed regularly the major individual fatty acids contained in D-004 (oleic, lauric, palmitic and myristic) from the food chain, it was rationale to believe that these compounds should not be toxic for humans. We did not found, however, information from formal studies of the long-term toxicity of these compounds (Entrez PubMed review up to July 2011). An old study referred the lack of oral single dose (15–19 g/kg of body weight) toxicity of oleic, lauric, palmitic, myristic, or stearic acids in rats (Sagepub, 1987). NOAEL values higher than >6000 and 5000 mg/kg have been reported for lauric acid administered orally to male rats for 18 weeks for palmitic acid administered to rats for 150 days, respectively (JECFA, 1997), while myristic acid did not induce a mutagenic response in either bacterial or mammalian systems in vitro, and that its use as food flavouring does not pose a health risk to humans (Burdock and Carabin, 2007). On the other hand, the regular intake of oleic acid with the diet, as occur in the Mediterranean diet has been associated with cardiovascular benefits rather with adverse effects (Faxén-Irving and Cederholm, 2010; Gillingham et al., 2010). This scenario encouraged the conduction of this study, since the specific composition of D-004 may determine its relative efficacy/toxicity. For more definitive conclusions, however, we are conducting a

**Table 3**
Effects of 1 year oral administration of D-004 on the rat blood biochemical parameters (mean ± SD).

| Treatment (mg/kg/day) | Creatinine (μmol/L) | Triglyceride (mmol/L) | Glucose (mmol/L) | ALT (U/L) | AST (U/L) | AChE (U) |
|---|---|---|---|---|---|---|
| *Males* | | | | | | |
| Control | 40.01 ± 7.75 | 1.16 ± 0.41 | 6.40 ± 0.94 | 43.44 ± 12.84 | 104.03 ± 24.84 | 0.37 ± 0.12 |
| 800 | 40.35 ± 6.27 | 1.20 ± 0.42 | 6.35 ± 1.18 | 41.11 ± 10.94 | 105.36 ± 20.87 | 0.39 ± 0.09 |
| 1500 | 41.68 ± 6.17 | 1.55 ± 0.94 | 6.65 ± 1.44 | 38.56 ± 10.48 | 98.06 ± 25.12 | 0.37 ± 0.09 |
| 2000 | 40.00 ± 8.07 | 1.21 ± 0.42 | 5.86 ± 0.95 | 40.12 ± 16.83 | 101.57 ± 27.29 | 0.39 ± 0.09 |
| *Females* | | | | | | |
| Control | 50.26 ± 9.11 | 1.28 ± 0.49 | 6.63 ± 1.32 | 32.18 ± 7.41 | 78.41 ± 16.61 | 0.45 ± 0.12 |
| 800 | 50.22 ± 7.44 | 1.30 ± 0.84 | 6.18 ± 1.02 | 32.85 ± 6.31 | 87.68 ± 16.67 | 0.47 ± 0.13 |
| 1500 | 49.60 ± 6.66 | 1.00 ± 0.37 | 5.91 ± 1.23 | 34.42 ± 15.13 | 84.42 ± 19.67 | 0.47 ± 0.12 |
| 2000 | 51.38 ± 5.13 | 1.21 ± 0.46 | 6.03 ± 1.06 | 30.69 ± 8.76 | 82.42 ± 18.86 | 0.46 ± 0.12 |

| Treatment (mg/kg/day) | Cholesterol (mmol/L) | AP (U/L) | Total Protein (g/dL) | Urea (mmol/L) | Creatine kinase (U/L) |
|---|---|---|---|---|---|
| *Males* | | | | | |
| Control | 1.75 ± 0.73 | 66.05 ± 26.03 | 6.25 ± 0.41 | 5.21 ± 0.82 | 428.42 ± 160.91 |
| 800 | 2.14 ± 1.00 | 61.55 ± 18.31 | 6.22 ± 0.43 | 5.41 ± 1.11 | 450.20 ± 140.14 |
| 1500 | 2.16 ± 1.23 | 63.25 ± 22.80 | 6.26 ± 0.47 | 4.91 ± 0.61 | 387.70 ± 166.78 |
| 2000 | 1.89 ± 0.80 | 68.00 ± 22.71 | 6.46 ± 0.51 | 5.03 ± 0.64 | 452.80 ± 139.86 |
| *Females* | | | | | |
| Control | 1.95 ± 0.53 | 25.58 ± 9.60 | 6.71 ± 0.40 | 5.06 ± 1.22 | 407.79 ± 204.11 |
| 800 | 1.94 ± 0.51 | 25.65 ± 16.36 | 6.60 ± 0.36 | 5.07 ± 0.58 | 357.55 ± 115.87 |
| 1500 | 1.92 ± 0.51 | 26.70 ± 13.04 | 6.58 ± 0.42 | 5.16 ± 0.97 | 344.90 ± 117.56 |
| 2000 | 1.97 ± 0.43 | 23.78 ± 6.12 | 6.55 ± 0.38 | 4.72 ± 0.95 | 380.17 ± 122.70 |

There were not significant differences between D-004-treated and control groups.
AChE, acetylcholinesterase; ALT, alanine aminotransferase; AST, aspartate aminotransferase., AP, alkaline phosphatase.

**Table 4**
Effects of 1 year oral administration of D-004 on the rat relative organ weights[a] (mean ± SD).

| Group (mg/kg/day) | Males | | | | | |
|---|---|---|---|---|---|---|
| | Liver | Right Kidney | Left Kidney | Heart | Thymus | Spleen |
| Control | 2.41± 0.21 | 0.32 ± 0.02 | 0.32 ± 0.02 | 0.26 ± 0.02 | 0.04 ± 0.01 | 0.15 ± 0.02 |
| 800 | 2.41 ± 0.24 | 0.32 ± 0.04 | 0.32 ± 0.04 | 0.25 ± 0.03 | 0.06 ± 0.04 | 0.14 ± 0.04 |
| 1500 | 2.42 ± 0.35 | 0.31 ± 0.05 | 0.31 ± 0.04 | 0.26 ± 0.02 | 0.04 ± 0.02 | 0.15 ± 0.03 |
| 2000 | 2.36 ± 0.17 | 0.31 ± 0.03 | 0.31 ± 0.03 | 0.25 ± 0.02 | 0.04 ± 0.02 | 0.14 ± 0.02 |
| | Lungs | Right Testis | Left Testis | Prostate | Right Epididymis | Left Epididymis |
| Control | 0.30 ± 0.04 | 0.23 ± 0.07 | 0.22 ± 0.08 | 0.26 ± 0.03 | 0.11 ± 0.02 | 0.11 ± 0.02 |
| 800 | 0.31 ± 0.04 | 0.25 ± 0.06 | 0.25 ± 0.06 | 0.23 ± 0.06 | 0.11 ± 0.02 | 0.11 ± 0.02 |
| 1500 | 0.33 ± 0.05 | 0.27 ± 0.05 | 0.27 ± 0.05 | 0.24 ± 0.05 | 0.11 ± 0.02 | 0.12 ± 0.05 |
| 2000 | 0.30 ± 0.06 | 0.26 ± 0.06 | 0.26 ± 0.06 | 0.27 ± 0.07 | 0.11 ± 0.02 | 0.11 ± 0.02 |
| | Seminal V. | LABC | Bulb. glands | Prep. glands | Right Adrenal | Left Adrenal |
| Control | 0.48 ± 0.10 | 0.28 ± 0.05 | 0.03 ± 0.01 | 0.04 ± 0.01 | 0.006 ± 0.002 | 0.006 ± 0.002 |
| 800 | 0.43 ± 0.09 | 0.25 ± 0.05 | 0.03 ± 0.01 | 0.03 ± 0.01 | 0.005 ± 0.001 | 0.005 ± 0.001 |
| 1500 | 0.42 ± 0.11 | 0.24 ± 0.05 | 0.03 ± 0.01 | 0.03 ± 0.01 | 0.005 ± 0.002 | 0.005 ± 0.002 |
| 2000 | 0.45 ± 0.13 | 0.26 ± 0.05 | 0.03 ± 0.01 | 0.03 ± 0.01 | 0.005 ± 0.001 | 0.005 ± 0.001 |

| Group (mg/kg/day) | *Females* | | | |
|---|---|---|---|---|
| | Liver | Right kidney | Left kidney | Heart |
| Control | 2.36 ± 0.28 | 0.29 ± 0.04 | 0.30 ± 0.05 | 0.29 ± 0.08 |
| 800 | 2.15 ± 0.14 | 0.26 ± 0.06 | 0.27 ± 0.03 | 0.26 ± 0.02 |
| 1500 | 2.28 ± 0.35 | 0.29 ± 0.04 | 0.29 ± 0.05 | 0.27 ± 0.02 |
| 2000 | 2.17 ± 0.14 | 0.29 ± 0.03 | 0.29 ± 0.03 | 0.26 ± 0.02 |
| | Thymus | Spleen | Lungs | Uterus |
| Control | 0.07 ± 0.02 | 0.18 ± 0.05 | 0.35 ± 0.06 | 0.21 ± 0.12 |
| 800 | 0.07 ± 0.02 | 0.17 ± 0.05 | 0.35 ± 0.06 | 0.16 ± 0.07 |
| 1500 | 0.07 ± 0.03 | 0.17 ± 0.04 | 0.34 ± 0.10 | 0.23 ± 0.09 |
| 2000 | 0.06 ± 0.02 | 0.18 ± 0.03 | 0.37 ± 0.04 | 0.23 ± 0.09 |
| | Right ovary | Left ovary | Right adrenal | Left adrenal |
| Control | 0.022 ± 0.008 | 0.031 ± 0.025 | 0.011 ± 0.003 | 0.010 ± 0.005 |
| 800 | 0.032 ± 0.041 | 0.032 ± 0.041 | 0.009 ± 0.003 | 0.009 ± 0.002 |
| 1500 | 0.037 ± 0.041 | 0.024 ± 0.010 | 0.011 ± 0.004 | 0.011 ± 0.004 |
| 2000 | 0.026 ± 0.014 | 0.025 ± 0.009 | 0.011 ± 0.003 | 0.011 ± 0.004 |

*Note:* There were not significant differences between D-004-treated and control groups.
Seminal V., seminal vesicles; LABC, levator ani bulbocavernosous; Bulb. glands, bulbourethral glands; Prep. Glands, preputial glands.
[a] Expressed as organ-to-body-weight percentage ratio.

**Table 5**
Non-neoplastic histopathological lesions observed following oral administration of D-004 for 1 year to Sprague Dawley rats[a].

| Observations | Males | | | | Females | | | |
|---|---|---|---|---|---|---|---|---|
| | Treatment (mg/kg/day) | | | | Treatment (mg/kg/day) | | | |
| | Control | 800 | 1500 | 2000 | Control | 800 | 1500 | 2000 |
| *Urinary system* | | | | | | | | |
| *Kidneys: Glomerulonephrosis* | 2/20 | 1/20 | 1/20 | 0/20 | 2/20 | 0/20 | 0/20 | 0/20 |
| *Kidneys: Cyst* | 0/20 | 0/20 | 0/20 | 1/20 | 0/20 | 0/20 | 0/20 | 0/20 |
| *Endocrine system* | | | | | | | | |
| *Adrenals: Peliosis* | 0/20 | 0/20 | 0/20 | 0/20 | 2/20 | 0/20 | 0/20 | 0/20 |
| *Pituitary: Hyperplasia* | 0/20 | 0/20 | 0/20 | 1/20 | 0/20 | 0/20 | 0/20 | 0/20 |
| *Pituitary: Congestion* | 0/20 | 1/20 | 0/20 | 0/20 | 2/20 | 2/20 | 1/20 | 0/20 |
| *Pituitary: Haemorrhagic* | 1/20 | 1/20 | 0/20 | 0/20 | 0/20 | 0/20 | 2/20 | 0/20 |
| *Reproductive system* | | | | | | | | |
| *Endometrial hyperplasia* | – | – | – | – | 2/20 | 0/20 | 0/20 | 3/20 |
| *Ovary: Cyst* | – | – | – | – | 1/20 | 0/20 | 1/20 | 1/20 |
| *Prostatitis* | 1/20 | 0/20 | 1/20 | 3/20 | – | – | – | – |
| *Testis: Atrophy* | 4/20 | 1/20 | 0/20 | 1/20 | – | – | – | – |
| *Epididymis: Atrophy* | 2/20 | 1/20 | 0/20 | 1/20 | – | – | – | – |
| *Respiratory system* | | | | | | | | |
| *Lungs* | | | | | | | | |
| *Histiocytosis* | 0/20 | 0/20 | 0/20 | 0/20 | 1/20 | 0/20 | 0/20 | 0/20 |
| *Granuloma* | 1/20 | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 |
| Total of animal with lesions | 7/20 | 3/20 | 1/20 | 5/20 | 8/20 | 2/20 | 4/20 | 3/20 |

[a] The following organs did not show lesions: liver, heart, spleen, thymus, thyroid, lymph nodes, bone marrow, larynx/trachea, bronchi, salivary glands, tongue, oesophagus, stomach, small and large intestine, pancreas, seminal vesicles, penis, vagina, urinary bladder, skeletal muscle, skin, eyes, Harderian glands, Zymbal gland, sciatic nerve, cerebrum, cerebellum and spinal cord.

2 years study of the long-term oral toxicity/carcinogenicity of D-004 in rats.

In conclusion, long-term oral treatment of rats with D-004 (800–2000 mg/kg/day) did not show evidences of D-004-related

**Table 6**
Neoplastic histopathological lesions observed following oral administration of D-004 for 1 year to Sprague Dawley rats.

| Observations | Males | | | | Females | | | |
|---|---|---|---|---|---|---|---|---|
| | Treatment (mg/kg/day) | | | | Treatment (mg/kg/day) | | | |
| | Control | 800 | 1500 | 2000 | Control | 800 | 1500 | 2000 |
| *Subcutaneous cell tissue* | | | | | | | | |
| *Malignant histiocytoma* | 0/20 | 0/20 | 0/20 | 0/20 | 2/20 | 0/20 | 0/20 | 0/20 |
| *Benign histiocytoma* | 1/20 | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 |
| *Reproductive system* | | | | | | | | |
| *Leiomyoma* | – | – | – | – | 0/20 | 0/20 | 0/20 | 1/20 |
| *Endometrial polyp* | – | – | – | – | 0/20 | 1/20 | 0/20 | 0/20 |
| *Hemolymphopoietic system* | | | | | | | | |
| *Thymoma* | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 | 1/20 | 0/20 |
| Pituitary gland | | | | | | | | |
| *Adenoma* | 0/20 | 0/20 | 1/20 | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 |
| Total of animals with lesions | 1/20 | 0/20 | 1/20 | 0/20 | 2/20 | 1/20 | 1/20 | 1/20 |

toxicity under the conditions of the study. The highest dose of D-004 tested (2000 mg/kg), therefore, should be considered as a non observable adverse-effect level (NOAEL) in rats.

## Conflict of Interest

The authors declare that there are no conflict of interest.

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