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Negative Evidence for Stachydrine or *Galeopsis ladanum* L. Seeds as the Causal Agents of Coturnism after Quail Meat Ingestion

Inigo Uriarte-Pueyo,[†] María Goicoechea,[§,∥] Ana Gloria Gil,[‡] Adela López de Cerain,*[,‡] Adolfo López de Munain,[§,∥] and María Isabel Calvo[†]

[†]University of Navarra, School of Pharmacy, Department of Pharmacy and Pharmaceutical Technology, Pamplona, Spain, [‡]University of Navarra, School of Pharmacy, Department of Nutritional Sciences, Physiology and Toxicology, Pamplona, Spain, [§]Hospital Donostia, Unidad Experimental, San Sebastián, Spain, and [∥]Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Spain

Quail poisoning is known to produce an acute myoglobinuric syndrome called coturnism. The cause of this syndrome is still unknown, although it has been postulated that *Galeopsis ladanum* L. seeds, in particular lipidic compounds or stachydrine, are responsible for this toxicity. Thus, we aimed to study the implication of this plant in coturnism in order to explore the physiopathology of the disease, especially with regard to stachydrine and lipidic compounds extracted from seeds. For this purpose, Wistar rats were fed with *G. ladanum* seed extracts or with quail meat. However, the rhabdomyolysis outbreak could not be reproduced in any case. Therefore, in view of our results and experimental conditions, seeds of *G. ladanum* and stachydrine do not appear to be the responsible agents of the myopathic outbreak. This conclusion is supported by the following facts: direct administration of extracts of seeds of *G. ladanum* or stachydrine produces no myotoxicity in rats; *G. ladanum* seeds are not toxic to quails and meat from quails fed *G. ladanum* seeds is not toxic to rats.

KEYWORDS: *Galeopsis ladanum*; rhabdomyolysis; betaines; stachydrine; coturnism; quail poisoning

INTRODUCTION

Acute rhabdomyolysis is a syndrome resulting from direct muscle injury as well as from damage to striated muscle, usually due to toxic, ischemic, infectious, inflammatory, or metabolic insults (*1*). A large number of drugs and toxins may induce myopathic changes, characterized by a great variety of symptoms or signs such as muscle weakness, pain, tenderness, and sometimes an asymptomatic elevated creatine kinase (CK) level (hyperCKemia). The clinical and pathological features depend on the causative agent and on a given susceptibility to a given compound. Some types of toxic myopathies could include necrotizing myopathies, mainly due to lipid-lowering drugs (fibrates and statins) (*2*−*5*), antimalarial drugs (*3*), thiol derivatives, alcohol, drug abuse, and mushrooms (*6*). Toxic myopathies are usually reversible after discontinuation of the offending agent (*7*).

A peculiar type of toxic myopathy is coturnism, described in many rural Mediterranean areas such as Algeria, France, Spain, Greece, or Italy (*8*−*12*). Most common symptoms of coturnism appear after 6−8 h of quail ingestion and include myalgia, stiffness, cramps, muscle weakness, and myoglobinuria associated with elevated plasma levels of aldolase, aspartate transaminase (SGOT), creatine kinase (CK), lactate dehydrogenase (LDH), and anuria or oliguria with azothemia. Duration of symptoms may vary from 1 to15 days (*10*).

The pathogenic basis of quail poisoning is attributed to genetic sensitivity (*13*−*16*) and/or a toxic effect (*17*) due to previous consumption of seeds by the quail (*9*, *16*). Seeds from two plants have been proposed as being responsible for the toxic effect in the literature: *Conium maculatum* (*18*−*20*) and *Galeopsis ladanum* (*10*). With regard to *Galeopsis ladanum*, Komarov et al. attributed toxicity to lipidic compounds of the seeds (*21*), whereas Aparicio et al. (*10*) pointed at stachydrine as the compound responsible for the disease.

The first rhabdomyolysis outbreak by quail meat ingestion described in Spain was in Congosto de Valdavia in 1999. Twenty patients suffered from rhabdomyolysis by ingestion of quails that fed on crops containing *Galeopsis ladanum* L. seeds (*10*). It was concluded that quails that eat *G. ladanum* seeds are toxic for human beings and that a plant betaine, probably stachydrine, could be the toxic agent involved in the outbreak. Nevertheless, this hypothesis needs experimental confirmation. Hence, this study aimed to examine the possible implication of stachydrine and/or other compounds, present in lipidic and/or aqueous extracts, of the seeds of *Galeopsis ladanum* L. in coturnism.

MATERIAL AND METHODS

Plant Material. Entire plant and seeds were collected in Congosto de Valdavia, Palencia, Spain. The plant was identified by Dr. R.Y. Cavero

*Corresponding author. Tel: +34 948 425 653. Fax: +34 948 425 652. E-mail: acerain@unav.es.

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and deposited in the herbarium of the Department of Plant Biology of the University of Navarra, Spain (herbarium number: PAMP 22874).

Dry powdered plant material (631 g of seeds and 194 g of leaves) was extracted by sequential cold maceration using 5 L of dichloromethane, ethyl acetate, methanol, and water.

Phytochemical Analysis. All extracts were subjected to preliminary phytochemical screening for the determination of major chemical groups by thin layer chromatography (TLC) (*22*) and high performance liquid chromatography coupled to UV detector (HPLC-UV). Polyphenol and betaine content were measured.

Isolation, Identification, and Quantification of Betaines. Betaines were isolated by preparative TLC, first with a mobile phase of $CHCl_3$/MeOH/NH_3 (5:4:1). After extracting them from plates with 0.1 M EtOH/HCl (9:1), betaines were completely purified by other preparativeTLC with a mobile phase of 0.1 M MeOH/HCl (9:1). Identification of the betaines was performed by Ultra performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry (UPLC-MS/MS) and ^{1}H nuclear magnetic resonance (NMR).

UPLC analyses were performed using a Waters Acquity Ultra Performance liquid chromatography system. Detection was carried out using Acquity PDA UV−vis detector coupled in series with an Acquity TQD tandem-quadrupole MS equipped with a ZSpray interface. The column used was a 150 mm × 3.9 mm i.d., 1.7 μm, Acquity BHE C_{18} (Waters, Barcelona, Spain) maintained at 25 °C, with a mobile phase flow rate of 0.4 mL/min. The mobile phase contained (A) methanol/5 mM ammonium acetate and (B) water/5 mM ammonium acetate. Gradient elution was used, starting at 10% A and rising linearly to 90% A over 10 min, then to 10% A over 3 min, followed by re-equilibration for 2 min. The injection volume was 1 μL. The instrument was operated using an API electrospray source in either the positive or negative ion modes. The ionization source parameters were as follows: 3.2 kV capillary voltage for positive mode and 2.8 kV for negative mode, 105 °C source temperature, and 300 °C desolvation gas temperature. The cone voltage was 40−80 V. Targeted MS/MS experiments were performed using a collision energy of 10−60 eV.

^{1}H NMR spectra were recorded on a Bruker 400 Ultrashield 400 MHz spectrometer. Five to ten milligrams of the isolated compounds were measured in DMSO-d_6.

Nonaqueous titration was necessary in order to quantify betaine content. Fifty grams of dry extract was dissolved in 200 mL of 95% glacial acetic acid (Panreac, Pamplona, Spain), and then 50 mL of acetic anhydride and 5 drops of indicator (crystal violet) were added. In order to calculate the percentage of betaines, this solution was titrated with 0.1 N perchloric acid in acetic acid (Panreac, Pamplona, Spain) until a color change occurred.

Quantification of Polyphenols. Total polyphenol content was measured using a new UV-spectrophotometric method with microplates (*23*).

Animals. This study was approved by the Ethics Committee on Animal Experimentation at the University of Navarra.

Rats. Seven-week-old (210−250 g) male Wistar Hannover rats were obtained from Harlan Iberica (Barcelona, Spain). On the day of arrival, the animals were weighed and randomly distributed into groups in polycarbonate cages with stainless steel covers, for a one-week period of acclimatization to the environmental conditions (12 h day/night cycle, temperature 22 ± 2 °C, relative humidity 55 ± 10%). They were provided with a standard diet from Harlan Iberica Spain and water ad libitum. Carbon dioxide (CO_2) was used for euthanasia.

Quails. Ten-week-old (100−120 g) male quails (*Coturnix japonica*) were obtained from Coto Valdorba (Sansoain, Navarra, Spain) and housed in groups of 3−4 animals per cage (70 × 90 × 100 cm^3), allowing a two-week acclimatization period. All animals were kept at a constant temperature (18 ± 3 °C) in a 12:12 h light/dark cycle and were provided with food and water ad libitum. Anesthetized animals were euthanized by exsanguination.

In Vivo Study Design. *Study of the Toxicity of G. ladanum Extracts on Rats.* The first part of the study aimed to analyze the possible toxicity of compounds from seeds of *Galeopsis ladanum* L.: stachydrine, lipidic compounds (dichloromethane extract), and polar compounds (aqueous extract). Animals were randomly distributed into five groups (10 animals per cage), and after one week of acclimatization, they were treated during three consecutive days by oral gavage. The rats received three doses of an extract of *G. ladanum* equivalent to 1.1 kg of seeds for a 75 kg adult human (yields of extraction: 42.6% dichloromethane extract and 2.7% aqueous extract). The stachydrine dose was calculated according to the information from Aparicio et al. (*10*). The control group received saline (10 mL/kg). Treated group 1 received the dichloromethane extract (6.4 g/kg). Treated group 2 received stachydrine (0.0015 g/kg). Treated group 3 received stachydrine (0.0015 g/kg) and the dichloromethane extract (6.4 g/kg). Treated group 4 received the aqueous extract (0.4 g/kg).

Study of the Acceptability and Influence of G. ladanum Seed-Based Diet on Quails. The aim of the second phase of the study was to determine the acceptability and influence of the *G. ladanum* seed-based diet on quails and to provide quail meat for the third phase of the study. Animals were randomly distributed into three groups (3 animals per cage) and fed ad libitum during 14 days. The control group was fed with standard fodder. Treated group 1 was fed with *G. ladanum* seeds. Treated group 2 was fed with standard fodder and *G. ladanum* seeds. The ingested food was weighed every 2−3 days.

An independent experiment with two groups of quails was performed in order to verify whether the *G. ladanum* seeds had any toxicity in quails. Animals were randomly distributed into two groups (11 animals per cage) and fed ad libitum during 14 days. The control group was fed with standard fodder. Treated group 1 was fed with *G. ladanum* seeds.

Study of the Toxicity of Meat from Quails Fed G. ladanum Seeds in Rats. The objective of the third phase of the study was to try to reproduce the myopathy outbreak in rats after quail meat ingestion and to confirm or reject the possible implication of *G. ladanum*. These animals were also used to determine the possible toxicity of *G. ladanum* seeds in quails by biochemical and histological analysis. Rats were randomly distributed into two groups (11 animals per cage) and treated during 3 consecutive days with a preparation of quail meat administered by oral gavage. These doses were equivalent to 310 g of quail meat each for a 75 kg human. The control group received quail meat fed on standard fodder (5 g/kg). The treated group received meat from quails fed on *G. ladanum* seeds (5 g/kg).

Behavioral Tests. *Rats.* The Irwin test (*24*) is a systematic and quantitative observational procedure for assessing the physiological and behavioral state of rodents. We paid special attention to the following indicative parameters of the myopathic process: locomotor activity, transfer arousal, body position, grip strength, limb tone, abdominal and body tone, equilibrium, type gait, and righting reflex. The animals were observed before every administration, 4 h after the administration, and before euthanization.

Quails. Quail behavior was recorded every day using a video camera over a period of 3 h.

Biochemical Analysis. The method used for blood extraction and processing was optimized using a previously described method (*25*). Anesthesia was induced by inhalation of 5% isofluorane (Forenew, Deutsche Abbott, Delkenheim, Germany), and it was maintained using a polycarbonate mask with 2.6% isofluorane. Blood samples were taken from the tail vein (0.5−1 mL) and by cardiac puncture (5−10 mL) in rats and quails, respectively, after anesthesia, placed into gel serum separator BD Microtainer tubes, and refrigerated at 4 °C for a minimum of 30 min until centrifugation. All samples were centrifuged at 4 °C for 15 min at 2778*g* (4000 rpm) in Heraeus Megafuge 1.0 R. The serum was analyzed in a Hitachi 911 spectrophotometer with creatine kinase liquid according to IFCC (*26*) and LDH optimade (*27*).

Histological Analysis. Gastrocnemius and soleus muscle samples were removed after euthanization and immediately frozen in isopentane cooled liquid for further analysis. Transverse 5 μm sections were cut and processed for hematoxilin and eosin (H&E), and modified Gomori histological techniques.

Nutritional Tests. The moisture percentage was obtained taking three samples of fodder and three samples of seeds of 5 g and drying them in a stove (102 °C) for 12 h upto constant weight. Total ashes have been quantified according to AOAC Method 920.153. The content in fats was determined by extracting the lipophilic compounds with petroleum ether in Soxhlet for 2 h. Protein content was calculated by Kjeldahl's procedure. The percentage of carbohydrates was calculated by the difference between 100 and the sum of the rest of the analyzed components.

At the beginning of the 20th century, Atwater proposed a few factors for calculating the caloric contribution of food, considering intestinal

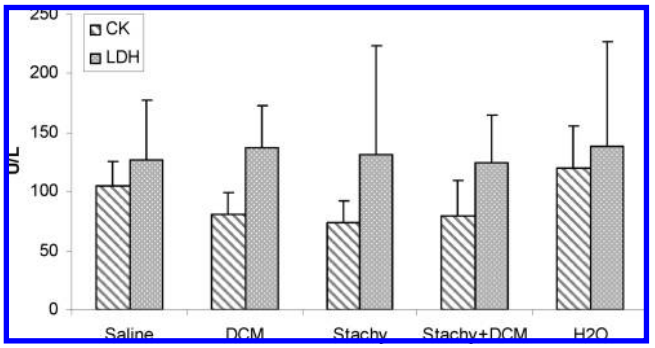

Figure 1. Biochemical results for the control group (saline), treated group 1 (dichloromethane extract, DCM), treated group 2 (stachydrine), treated group 3 (stachydrine + dichloromethane), and treated group 4 (aqueous extract, H_2O).

incomplete absorption (*28*). The caloric contribution of fodder and *G. ladanum* seeds has been obtained by applying Atwater's factors: 9 kcal/g for fats, 4 kcal/g for proteins, and 4 kcal/g for carbohydrates.

Statistic Analysis. Statistical analyses were performed with the SPSS v15.0 program. Body weight values between the different groups were compared by a Kruskall−Wallis test. CPK and LDH values between groups were compared by Student's *t*-test.

RESULTS AND DISCUSSION

Phytochemical Analysis. The study of the composition of *G. ladanum* seeds by TLC and HPLC-UV shows that the principal compounds are fatty acids, betaines, phenolic acids, and saponins (*29*).

A very low content in polyphenol compounds ($< 0.722\ \mu g/mL$) was detected in dichloromethane and aqueous extracts. Polyphenols were present principally in ethyl acetate and methanolic extracts. The betaine content of the *G. ladanum* seeds was 0.39%.

Two betaines were isolated from *G. ladanum* seeds (compounds **1** and **2**). The analysis by UPLC-MS/MS of a standard of stachydrine confirmed the identity of compound **1** as stachydrine, with a molecular ion $[M]^+$ (143.80 *m*/*z*) (*30*). Compound **2** presented a molecular ion at *m*/*z* 103.98 and two daughter ions at *m*/*z* 45 and 60. The molecular ion and fragmentation patterns could correspond to choline (*31*). In order to confirm the structure of compound **2**, ^1H NMR and ^{13}C- NMR were preformed. The results of these analyses were as follows: ^1H NMR (400 MHz, D_2O) δ ppm = 3.07 (6H, s), 3.22 (3H, s), 3.39 (2H, t, $J = 4.4$ Hz), 3.93 (2H, t, $J = 2.8$ Hz), 4.69 (s). ^{13}C NMR (400 MHz, D_2O) δ ppm = 54.32, 56.04, 67.79. NMR confirmed the identity of compound **2** as choline (*32*, *33*).

Toxicity of *G. ladanum* Extracts in Rats. The first part of the study aimed to analyze the toxicity of the possible toxic compounds of *G. ladanum* seeds: stachydrine, lipidic compounds (dichloromethane extract), and polar compounds (aqueous extract) in rats.

Before beginning this assay, the different methodologies to be used were validated. Blood extraction and processing were optimized for the correct determination of creatine kinase (*25*). The most important parameters in myopathic processes were characterized after the administration of paraphenylenediamine (PPD) (a well-known myotoxic compound) in a single dose of 60, 80, and 100 mg/kg (information not shown).

The administration of different plant extracts showed no symptomatological (Irwin test) or histopathological alterations. A biochemical study detected no significant differences with regard to the control group (**Figure 1**). Direct oral administration of *G. ladanum* extracts presented no myotoxicity in rats.

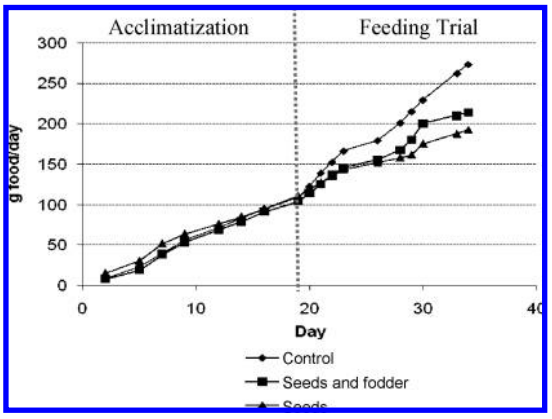

Figure 2. Ingested grams of food accumulated per day of each group.

The present study was undertaken with seeds collected between 2005 and 2007, with no cases of rhabdomyolysis reported at that time. It is known that environmental stress involves the adaptation of plants that lead to accumulated metabolites (*34*, *35*). These metabolites include nitrogen-containing compounds (proline, other aminoacids, quaternary amino compounds, and polyamines) and hydroxyl compounds (sucrose, polyols, and oligosaccharides) (*36*). Indeed, it has been published that stachydrine metabolism varies depending on osmolarity conditions. Catabolism is active at low osmolarity but reduced at high osmolarity (*37*). This consideration led us to hypothesize that a compound from stachydrine metabolism alone or in combination with others might be responsible for the toxicity, as commercially obtained stachydrine did not produce any deleterious effects in rats. It could be also postulated that a mycotoxin-producing fungus in combination with *G. ladanum* seeds could exert such toxicity. There are wide varieties of toxigenic species that contaminate human food and animal feed such as *Aspergillus*, *Penicillium*, or *Fusarium* species. However, we failed to find references associated with myotoxic effects.

Toxicity of *G. ladanum* Seeds on Quails. The aim of the second phase of the study was to determine the acceptability and influence of a *G. ladanum* seed-based diet on quails and to provide quail meat for the third phase of the study.

First, in order to determine the acceptability of the diet, the quantity of ingested food was evaluated in each group (**Figure 2**). Groups fed with seeds consumed a minor quantity of food with regard to the control group. A loss of weight was detected in treated groups with regard to the beginning of the treatment but with no significant differences. To determine if the minor ingestion of food and the loss of weight detected were due to the caloric contribution, the basic nutritional composition of fodder and *G. ladanum* seeds were analyzed (**Table 1**). Then, according to the nutritional analysis and the quantity of ingested food, the kilocalories consumed by each group were calculated (**Table 2**). The seeds presented a high content in fats and therefore a major caloric contribution. Nevertheless, the groups fed on seeds consumed a minor quantity of calories than the control group, and this explains the detected weight loss.

Ingestion of *G. ladanum* seeds showed no behavioral or histopathological changes in quails. Only one quail presented hepathomegalia as a result of granulomatous hepatitis, unrelated to myotoxic damage. No significant differences were detected in CK and LDH.

Toxicity of Meat from Quails Fed on *G. ladanum* Seeds in Rats. The objective of the third phase of the study was to try to reproduce the myopathy outbreak in rats after quail meat ingestion and to confirm or reject the implication of *G. ladanum*

Table 1. Nutritional Analysis of Fodder and *G. ladanum* Seeds

	%		kcal/g	
	fodder	seeds	fodder	seeds
dampness	7.17 ± 0.03	3.53 ± 0.07		
fats	5.01 ± 0.13	15.77 ± 0.43	0.45	1.42
proteins	21.94 ± 0.22	17.47 ± 0.24	0.88	0.70
ash	5.45 ± 0.02	4.82 ± 0.04		
carbohydrates	60.43 ± 0.32	58.41 ± 0.43	2.42	2.34
kcal/1 g			3.75	4.46

Table 2. Energy Consumed by Each Group

	g food/day	kcal/day	% loss of weight
control	11	41.20	0.8 ± 2.2
seeds	5.9	26.28	8.2 ± 6.4
seeds and fodder	8.8 (3.4 fodder + 5.4 seeds)	36.80	6.3 ± 4.3

on the myotoxic insult. For this purpose, groups of 11 rats were dosed with quail meat that had been prepared in a formula that could be dosed by oral gavage.

Administration of meat from quails fed on *G. ladanum* seeds showed no behavioral (Irwin test) or histopathological changes in rats. No alteration was detected in the biochemical analysis (CK and LDH) of rats fed on supposedly toxic quail meat (**Figure 3**).

G. ladanum seeds might not be responsible for coturnism. Aparicio et al. (*10*) concluded that quails that eat *G .ladanum* seeds were toxic for human beings and that a plant betaine, probably stachydrine, could be the toxic agent involved in the outbreak. However, some limitations may arise from the work, such as the number of rats used in the experiment, which was limited. Moreover, CK levels were not statistically different between control and treated rats because of large variability. Finally, data regarding the quantity of seeds ingested by quails, clinical symptoms, or histological features of treated rats was absent. In our study, we used a parametric design with a large number of animals, we performed histological analysis, and we used a better method for the determination of CK with less variability. The multiple dose administrated to rats is equivalent to the ingestion of approximately 0.9 kg of quail meat (7−10 quails) for an adult of 75 kg, a high enough dose so as to be able to detect possible toxicity.

The pathogenesis of quail poisoning is not known. Some authors have suggested that an enzymatic defect in skeletal muscle fibers might play a role in the etiology of this disease, on the basis of the fact that it affects only some sensitive individuals that have ingested quails (*16*) and that some persons have referred familial occurrence (*9*). Moreover, it is known that muscular exertion before or after the meal aggravates and accelerates the manifestations (*15*, *38*). No defects in several muscle glycolitic, mitochondrial, and lypolitic enzymes were found in 2 out of 10 patients affected with coturnism, although these 2 patients did not report a family history of quail poisoning (*9*). Therefore, this hypothesis remains to be definitely confirmed or discarded. In any case, this would be a human condition that cannot be reproduced in animal models and consequently could not be addressed in this study.

With respect to the toxic agents, previous reports have speculated that a toxin or an alkaloid contained in seeds eaten by the quails may be involved, with hemlock (*Conium maculatum*) or *Galeopsis ladanum* L. being possible candidates. There is evidence that coniine, an alkaloid of *Conium maculatum*, is toxic to quails (*Colinus virginianus*) and that it was detected in bird skeletal muscle and liver 7 days after treatment (*39*). But, to our knowledge, there is no evidence of the implication of hemlock alkaloids

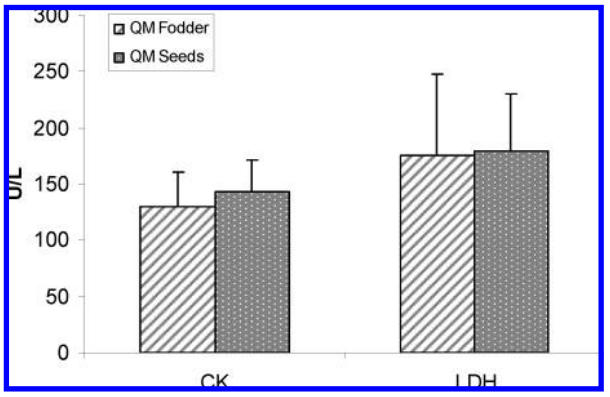

Figure 3. Biochemical results for the control group, meat from quail fed on fodder (QM fodder), and treated group, meat from quail fed on *G. ladanum* seeds (QM Seeds).

on human rhabdomyolysis or experimental work testing this hypothesis. The presence of this plant was not referred to in the publication regarding the Spanish myopathic outbreak in Congosto de Valdavia (*10*); moreover, the *C. maculatum* plant was not observed when *G. ladanum* seeds were collected in Congosto de Valdavia for this study. With respect to *Galeopsis Ladanum* L. seeds, two betaines, representing 0.39% seed content, could be isolated, with stachydrine being one of them. Nevertheless, rhabdomyolytic action could not be reproduced in animals with pure stachydrine or different plant extracts at doses equivalent to very high human exposure. Although phytochemical characterization has been very exhaustive and the experimental animal design carefully planned, different species sensitivities or factors that may influence alkaloid composition of seeds cannot be completely discarded as they are impossible to control.

In view of our results, we can conclude that neither stachydrine nor *G. ladanum* seeds appear to be the agents responsible for the myopathic outbreak under these experimental conditions. This conclusion is supported by the following facts: (a) direct administration of 3 doses of stachydrine (1.5 mg/kg) is not toxic to rats; (b) extracts of *G. ladanum* seeds produce no myotoxicity in rats; (c) *G. ladanum* seeds are not toxic to quails; and (d) meat from quails fed *G. ladanum* seeds is not toxic to rats.

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