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Effects of dietary *Crotalaria pallida* seeds on the health and performance of laying hens and evaluation of residues in eggs

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ABSTRACT

The effect of three dietary concentrations of *Crotalaria pallida* (*C. pallida*) seeds (0, 1, 2, and 3% w/w) of their normal diet were investigated in commercial laying hens during a 35 day feeding trial. All concentrations of *C. pallida* decreased body weight and feed intake ($P < 0.05$). Egg mass production and average egg weight were decreased by feeding of ≥2% *C. pallida* seeds ($P < 0.05$). All concentrations of *C. pallida* increased relative lung weight and serum activity of ALT, AST and LDH ($P < 0.05$); 3% *C. pallida* seeds decreased liver weight ($P < 0.05$). Analysis of the *C. pallida* seeds for dehydropyrrolizidine alkaloid content detected usaramine and its *N*-oxide at a total alkaloid concentration of 0.18% (dry weight). Usaramine was also detected in the eggs of all hens fed *C. pallida* seeds.

1. Introduction

Pyrrolizidine alkaloids (PAs) are a large group of compounds found in more than 6000 plant species (Chen et al., 2010). The 1,2-dehydropyrrolizidine alkaloids (DHPAs) are considered most toxic and are known to cause intoxication in animals and humans (Chen et al., 2010; Mattocks, 1986). Most of the plants capable of causing toxicosis in animals and humans belong to the genera *Senecio*, *Heliotropium*, *Symphytum* and *Crotalaria* (Edgar et al., 2011). *Crotalaria spp*. are commonly known as rattlebox or crotalaria, and are grown as cover crops on sandy soils. *Crotalaria spp*. have been shown to contain "monocrotaline-type DHPAs", including the highly toxic monocrotaline (Pilbeam et al., 1983) as well as fulvine, retusamine, and trichodesmine (European Food Safety Authority, 2011). *Crotalaria spp*. may also contain "senecionine-type DHPAs" (e.g. integerrimine, jacobine, retrorsine, senecionine, seneciphylline) which are particularly common in *Senecio spp*. (European Food Safety Authority, 2011). Poisoning by crotalaria has been reported in horses (Rose et al., 1957), rats (Copple et al., 2006), donkeys (Pessoa et al., 2013), sheep (Nobre et al., 2005), chickens and pigs (Hooper and Scanlan, 1977). DHPAs may also be transferred to animal products (meat, milk, eggs and honey), causing an additional exposure risk for humans (Edgar and Smith, 2000; European Food Safety Authority, 2011).

Crotalaria pallida Aiton (*C. pallida*, smooth crotalaria, striped crotalaria, smooth rattlebox) is a less known species that has been reported to be toxic for chickens (Diaz et al., 2003) and goats (Hernández and Canchila, 2001). *C. pallida* is a species native to Africa and usually grows in warm, open areas and in arid and semiarid regions (Fonseca et al., 2006). *C. pallida* is also used as green manure in rotation with animal feed or human food crops (Uratani et al., 2004). Consequently, there is potential for weedy re-growth that could lead to contamination of the food crop with seeds and other plant-parts of *C. pallida*. The present study investigated the effect of contamination of chicken feed by seeds from *C. pallida* on the health and productivity of the hens, and on the potential for transfer of toxic DHPAs into the human food chain via the eggs.

2. Materials and methods

Forty 32-week-old commercial laying hens (ISA Babcock Brown) were placed in individual wire cages in an environmentally controlled room at 20 °C with artificial light provided 14 h per day. The hens received feed and water *ad libitum*. The experiment was conducted following the welfare guidelines of the Poultry Research Facility, College of Veterinary Medicine, National University of Colombia and was approved by the local ethics committee. A completely randomized experimental design was employed consisting of four experimental groups with 10 hens per group. The four treatment diets were a commercial layer diet mixed with dried and ground (>86% of particles <20 mesh) *C. pallida* seeds. In addition to a negative control Group 1 receiving commercial layer diet, Groups

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2, 3 and 4 received the same diet but contaminated with 1%, 2% or 3% (w/w) *C. pallida* seeds respectively. Seeds were collected from specimens in a single field, positively identified as *C. pallida* by botanists of the Colombian National Herbarium of the National University of Colombia.

The diets were fed for 5 weeks. Feed intake, egg mass production (g/hen), egg weight and number of eggs laid were monitored at weekly intervals. Hens were individually weighed at the beginning of the experiment and at days 7, 14, 21, 28, and 35. Samples of approximately 3 mL whole blood were collected from each hen by venepuncture of the main brachial vein (*vena brachialis*) on days 0, 14 and 28 of the experiment. After centrifugation of the blood, serum activities of aspartate aminotransferase (AST, P/N 41293), alanine aminotransferase (ALT, P/N 41282), lactate dehydrogenase (LDH, P/N 41222), gamma-glutamyltransferase (GGT, P/N 41293), and creatine kinase (CK, P/N 41250) were determined by means of a kinetic ultraviolet spectrophotometric method, using commercially available kits (Spinreact, Girona, Spain).

Eggs were collected the day before the start of the experiment (day 0) and afterwards at weekly intervals (days 7, 14, 21, 28, and 35) for the analysis of DHPA content. Four or five eggs per dietary treatment were cracked open, their contents combined and homogenized and then freeze dried. In addition to these sampling times, eggs were also collected from the remaining hens 1 week after the end of the experiment and were analyzed for DHPAs.

At the end of the experiment, six hens from each dietary treatment were humanely euthanized in a CO_2 chamber and examined for gross lesions. The liver, lungs, heart, spleen, kidneys, proventriculus, gizzard and pancreas from each bird were removed and weighed. Samples of liver and lung were fixed in neutral buffered 10% formalin for 24 h, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin for histological examination.

2.1. Sample extractions for analysis of DHPAs

In duplicate, *C. pallida* ground and homogenized seed material (0.100 g) was extracted with 4.0 mL of 0.05M H_2SO_4 in a 15 mL screw cap glass tube by mechanical rotation for 1 h. The samples were centrifuged for 10 min (2500× *g*). A 0.2 mL aliquot of the acid extract was applied to solid phase extraction columns (Phenomenex, Strata SCX, 200 mg, pre-rinsed with 2 mL methanol, 2 mL water and 1 mL 0.05 M H_2SO_4). The columns were rinsed with 4 mL water and then 4 mL methanol. The alkaloid fraction was then eluted with 3 mL ammoniated methanol (10% saturated ammoniated methanol in methanol). The samples were evaporated to dryness under a flow of nitrogen at 60 °C and 2.0 mL of 50% methanol (0.1% formic acid) was added to reconstitute the samples. Samples were then analyzed for DHPAs by HPLC-(esi+)MS and MS/MS. Alkaloids were identified by comparison of retention time and MS/MS spectra with those from standard samples obtained from the Poisonous Plant Research Laboratory private collection of DHPAs.

In a similar manner, the dried egg samples (0.250 g) were extracted with 5.0 mL of 0.05M H_2SO_4 in a 20 mL screw cap plastic tube by mechanical rotation for 1 h. Acetonitrile (5.0 mL) was added to aid in protein precipitation and the samples were then mixed for an additional 15 min. The samples were centrifuged for 10 min (2500× *g*). A 4.0 mL aliquot of the acid/acetonitrile extract was applied to solid phase extraction columns (Phenomenex, Strata SCX, 200 mg, pre-rinsed with 2 mL methanol, 2 mL water and 1 mL 0.05 M H_2SO_4). The columns were rinsed with 4 mL water and then 6 mL methanol. The alkaloid fraction was then eluted with 5 mL ammoniated methanol. The samples were evaporated to dryness under a flow of nitrogen at 60 °C and 0.50 mL of 5% methanol was added to reconstitute the samples. Samples were filtered through a 0.25 μm syringe filter prior to analysis of DHPAs by HPLC-MS.

2.2. HPLC-MS analysis of DHPAs

The HPLC-(esi+)MS system included an Agilent 1260 binary pump, an Agilent 1260 autosampler, a Synergi Hydro-RP column (Phenomenex, 150 × 2.0 mm, with guard column) coupled to a Velos Pro linear ion trap mass spectrometer (ThermoScientific) and a heated electrospray ion source (HESI). A gradient mixture of acetonitrile (A) and 0.1% formic acid (B) was used for the mobile phase at a flow rate of 0.4 mL/min. The timed gradient elution conditions were: 3% A (0–2 min); 3–70% A (linear gradient 2–15 min); 70–3% A (15–18 min), and back to 3% A (18–25 min). For the seed analyses, the mass spectrometer used single stage MS detection with a scan range of *m/z* 200–800. As the *C. pallida* seed material was found to contain usaramine as the major alkaloid, only usaramine was targeted in the dried egg sample analyses using selected MS/MS analysis. For the analysis of dried egg samples the mass spectrometer was set to monitor daughter ions (*m/z* 100–400) from the parent ion (*m/z* 352.2) in MS/MS mode using a relative collision value of 35. Sample injection size was 5 μL for all analyses. Quantitative analyses were made by area under the curve of the selected ion trace *versus* a calibration curve prepared from a standard solution of usaramine (>95%, Poisonous Plant Research Laboratory collection). For plant analyses the calibration standards were prepared by serial dilution to give standard concentrations of 6.5, 3.25, 1.63, 0.81 and 0.40 μg/mL and using selected ion trace (*m/z* 325) for the protonated usaramine ($R^2 = 0.9993$ for linear regression over complete range of standards). For the analyses of dried egg samples the calibration standards were prepared by serial dilution to give standard concentrations of 125, 62.5, 31.2, 15.6, 7.8, 2.0, and 0.5 ng/mL and using combined selected ion trace (*m/z* 120, 138, 276, 324) for usaramine ($R^2 = 0.9998$ for linear regression over complete range of standards). Limit of quantitation (LOQ) was estimated to be 0.5 ng/mL by a S/N ~ 10 in sample solutions with a consequent calculated LOQ of 2 ng/g in dried egg samples.

2.3. Statistical analysis

Except for descriptions of gross and histological lesions, data for response variables serum enzymes and relative organ weights were subjected to analysis of variance (ANOVA) for a complete randomized design, using Statistix® for Windows version 9 (2008). Variable means from treatments showing significant differences in the ANOVA were compared using Tukey's test. Statements of significance are based on $P < 0.05$. The effect of the *C. pallida* treatment on performance parameters measured at weekly intervals was estimated by analysis of covariance for repeated measurements (SAS Institute, 2008; GLM procedure) using a mixed linear model with class variables *C. pallida* level and time and their interaction.

3. Results

Analysis of the *C. pallida* seeds by HPLC-MS for PAs resulted in the detection of usaramine and its corresponding *N*-oxide (Fig. 1). The concentration of usaramine was found to be 0.16% as the free base, and an estimated 0.02% as the *N*-oxide, for a total DHPA content of about 0.18% (dry weight basis). The estimated daily usaramine dose received by the hens fed 1, 2 and 3% *C. pallida* seeds was 0.54, 0.99, and 1.41 mg/kg, respectively.

No differences in the body weight recorded at weekly intervals were seen at the beginning of the experiment or at day 7 (Fig. 2). However, at days 14 and 21 of the experiment the hens receiving 2 and 3% *C. pallida* seeds had lower body weights than the controls ($P < 0.05$) and at days 28 and 35 all hens receiving *C. pallida* seeds had lower body weights ($P < 0.05$). At the end of the experiment, differences in body weight were found among all experimental groups ($P < 0.05$). At this stage, the body weights of the hens