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Short communication

CLINICAL OBSERVATIONS AND SAFETY PROFILE OF ORAL HERBAL PRODUCTS, *SOUROUBEA* AND *PLATANUS* spp; A PILOT-TOXICOLOGY STUDY IN DOGS

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This pilot-study evaluated the toxicity and safety profile of two herbal products *Souroubea spp* Botanical Blend (SSBB) and *Platanus* Tree Bark (PTB) after oral administration to dogs at elevated doses for 28 days. SSBB and PTB botanicals are the major active ingredients of Sin Susto™, a novel natural product for the treatment of anxiety in dogs. Three healthy female dogs were administered elevated doses of either SSBB, PTB or a placebo and then monitored for the occurrence of any systemic and local adverse events. Data from this pilot-study revealed that SSBB and PTB had no untoward effects on the health of dogs and were deemed safe which enabled the design and execution of a larger controlled target safety and toxicology study for Sin Susto™.

Key words: anxiety, dogs, *Souroubea* spp, *Platanus*, Sin Susto™

INTRODUCTION

Anxiety and aversion to loud sounds (noise aversion) are common behavioural problems in companion animals, particularly dogs. These behaviours are associated with fear responses, such as cowering, freezing, scanning, seeking out the owner, and attempting to escape. In some cases, anxiety may lead to property damage, dog injury, disruption of the owner-pet bond and potential pet abandonment or even euthanasia [1,2]. If left untreated anxiety may have a negative impact on overall animal health and reduce their lifespan [3]. Current treatments for anxiety and noise aversion in veterinary medicine include pharmacotherapy (benzodiazepines, phenothiazines, tricyclic antidepressants (TCAs) selective serotonin reuptake inhibitors (SSRI) behavioural management, systematic desensitization [1] and use of neutraceuticals [4].

Sin Susto™ (Bioniche Animal Health., Belleville, ON, Canada) is a herbal natural anxiolytic product intended to prevent or reduce both anxiety and stress in dogs. Sin

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Susto™ is formulated as a chewable tablet containing a blend of natural botanical ingredients: *Souroubea spp.* Botanical Blend (SSBB) and *Platanus spp.* Tree Bark (PTB). SSBB is known and accurately defined as "leaf and stem growth" of the neotropical vines: *Souroubea sympetela* Gilg. *or Souroubea gilgii* V.A. Richt. (Marcgraviaceae) or any mixture of both. PTB is defined as the shed bark of *Platanus occidentalis* L. (American Sycamore) or *Platanus acerfolia* Aiton Wild. (London Planetree) (Plantanaceae) or any mixture of these.

MATERIALS AND METHODS

Preparation and characterization of SSBB and PTB

The biochemical screening of the genus *Souroubea* spp. and *Platanus* spp. by the Biology and Chemistry Department at the University of Ottawa has not demonstrated any significant differences in the levels of the pentacyclic triterpenoids between the two species in each genus. The leaves and stems of *Souroubea sympetala* (UNA voucher no. JVR 12823) and *Souroubea gilgii* (UNA voucher no. JVR 12844 and JVR 12894) samples used in this pilot study were collected in Costa Rica. The *Platanus occidentalis* and *Pacerifolia* (OTT voucher no. 19608/9) samples were collected in Ottawa, Ontario, Canada. SSBB and PTB were prepared by drying in a herb drier at 45°C, thereon grinding the dried herbal material to a fine powder that passes through a 0.25 mm screen and thoroughly blending together. In both, SSBB and PTB the betulinic acid (BA) is considered to be the major active component. Both species were analysed for BA content by HPLC/MS methods as previously described [5]. BA possesses anxiolytic properties at low doses (1 mg/kg) [6] and has potential benefits in cancer therapy [7,8] reduction of HIV replication [9,10], inflammation [11] and bacterial infections at much higher doses (50-500 mg/kg) [12].

The primary objective of this pilot study was to assess the safety of each active herbal ingredient (AHI) SSBB and PTB at elevated doses after daily administration for 28 days in dogs.

Each one of the test articles (placebo, SSBB and PTB) were mixed with the double distilled water, commercial pet food (Eukanuba, Mfg230812 lot # 11134175), flour and flavour additive (Flavour Pal-beef flavour #U10K140) at the same ratio and provided in the form of a dog treat (dog biscuit). Test articles were tested for triterpene levels (including BA) at the University of Ottawa using high-performance liquid chromatography with tandem mass spectrometric detection for the quantitative determination (HPLC-MS-QTRAP). Briefly, biscuit samples were crushed in a blender and 2 g of crushed material was used for extraction A with 250 ml ethyl acetate using a Soxhlet apparatus for 2h. The extracts were dried by rotatory evaporation at 45°C, re-dissolved in ethyl acetate and dried at room temperature. Extracts were solubilized in 40 ml methanol by sonication for 10 minutes and filtered through 0.22 micron

PTFE filter. One microliter was injected through an autosampler for HPLC-MS-MS analysis. Triterpene contents were quantified by AB-SCIEX 3200 HPLC-MS-QTRAP. Method conditions: Column=Kinetex 2.6 micron, 100x2.00 mm Phenomenex; Mobile Phase=A-Water, B- Acetonitrile; Flow rate 0.4 mL/min; Column thermostat temperature +45°C; Separation: Linear Gradient+30-100% B in 18 min; Calibration curve was generated by injection of 1-200ug/ml purified compounds from Sigma-Aldrich or prepared by preparative HPLC. Analysis revealed that SSBB and PTB samples contained 0.22 (±0.034) mg/g and 0.68 (±0.016) mg/g of BA, respectively. The test articles were supplied in identical labelled packages by Bioniche Animal Health and identified as Placebo, SSBB and PTB with respective lot numbers. The appearance and characteristics of the products were visually similar. Upon receipt at the study site all test articles were stored in a freezer at -20°C to prevent deterioration. The study investigator was not blinded for experimental products. The technicians feeding and managing the dogs as well as laboratory personnel performing analysis were blinded for experimental treatments.

Study design

The study protocol was developed by 3 study participants (Villalobos, Baker and Arnason) reviewed and approved by the University of Ottawa Animal Care Committee (protocol #BL-146) and by the Ministry of Science and Technology-National Technical Committee on the Use of Laboratory Animals (PRONASEA), Costa Rica. The study was designed to avoid or minimize any discomfort, distress or pain to the animals during the course of the experiment. The experimental design included an acclimatization period of 16 days (Days-16 to -1), test/dosing period of 28 days (Days 0-28) and post-dosing monitoring period of 14 days (Days 29-42) (Table 1).

Three mixed-breed female dogs from a local shelter (Human Society, San Jose, Costa Rica) were randomly selected and enrolled in the study. Dogs enrolled in the study fulfilled inclusion criteria (normal hematological, biochemical and urine analysis, approximately 2.5 years old, body condition score 1-3 [13], and without any clinical signs of disease. The study site was the Veterinary Clinic Bethel Medical, San Jose, Costa Rica. Prior to the start of the study the dogs were acclimatized for 16 days during which time they underwent clinical examination (Days-16, -6 and -1), blood and urine workup (Day-1). In addition, dogs were treated for internal (gastrointestinal) and external parasites (fleas and ticks) at the beginning of the acclimatization period (Day-16). During the acclimatization dogs were fed with placebo biscuits twice daily (3 biscuits in the morning and 3 biscuits in the evening- approximately 10h apart).

Administrations of test articles SSBB, PTB and placebo started on Day 0 and were performed twice daily for 28 consecutive days with the total daily dose split into half and administered orally approximately 10h apart. The minimal effective dose is estimated to contain 1mg of BA/kg of body weight; therefore biscuits from both SSBB and PTB were formulated to contain 2 mg/g of BA.

Table 1. Pilot-toxicology study schedule of events for dogs receiving SSBB, PTB, or placebo twice daily

Period	Day	Body weight	Physical exam	Blood sample[a]	Uninalysis[b]	Fecal exam	Fasted	Treatment[c]
Acclimation (Days -16 to -1)	-16		●			●		Placebo 1x daily
	-6					●		
	-1		●					
Treatment Phase (Days 0-28)	0	●	●	●	●	●	●	Treatment 2x daily
	1	●	●	●	●		●	
	3	●	●	●	●		●	
	7	●	●	●	●		●	
	14	●	●	●	●		●	
	28	●	●	●	●		●	
Post-treatment Period (Day 29 to 42)	35	●	●	●	●		●	None
	42	●	●	●	●		●	

[a]Blood samples obtained between 8:30 am and 9:30 am; [b]Urine samples obtained between 6:00 am and 7:00 am; [c]Treatment administered at 9:30 am and 6:00 pm

RESULTS AND DISCUSSION

Dog #1 (7.2 kg) received three SSBB treats in the morning and three SSBB treats in the evening. Each SSBB treat contained 4.2g SSBB (8.4mg BA) which corresponded to seven times the recommended treatment dose of SSBB (7 mg BA/kg/day). Dog #2 (9.8kg) also received three placebo treats in the morning and three placebo treats in the evening, which contained no SSBB or PTB (0 mg BA/kg/day). Lastly, Dog #3 (17.5kg) received four PTB treats in the morning and three PTB treats in the evening, which was eight times higher than the daily recommended dose of PTB (8 mg BA / kg/day). Each PTB treat contained 1g PTB with 20 mg of BA.

Blood and urine samples were collected on days 0, 1, 3, 7, 14, 28, 35 and 42. Sampling was performed in the morning on each collection day. Dogs were fasted for 14 to 16 hours prior to sample collection. In addition, on the sampling day the dogs were weighed and subjected to physical examination (temperature, pulse, heart rate, respiratory rate and capillary refill time).

Dogs did not receive any concurrent medications during the course of study (Days 0 to 42).

The blood samples were analyzed for the following parameters: urea, creatinine, red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count,

segmented neutrophil count, lymphocyte count, eosinophil count, monocyte count, platelets, calcium, phosphorus, magnesium, glucose, aspartate transaminase, glutamic pyruvic transaminase, alkaline phosphatase, gamma-glutamyl transpeptidase, total protein, albumin, globulin, A:G ratio, bilirubin, total cholesterol, chloride, sodium, potassium, total T3, free T4, cortisol, ACTH, TSH, and neutrophil:lymphocyte ratio. The urine samples were analyzed for the following: colour, turbidity, specific gravity, pH, albumin, glucose, ketones, nitrites, ascorbic acid, bilirubin, urobilinogen, blood, leukocytes, red blood cells, epithelial cells, mucus filament, crystals, amorphous sediment, and bacteria. During the course of study dogs did not participate in any other experimental trials.

Throughout the study, the dogs were kept in clean cages, were exercised and monitored daily for any systemic or local changes. They were fed a diet of Purina® Pro Plan® and had unrestricted access to fresh water.

During the test/feeding stage of the study (Days 0 to 28) and 14 days follow-up (Days 29-42), all three dogs exhibited normal behaviour and there was no clinical evidence of any local or systemic adverse reactions. Hematology, biochemistry and urinalyses results from all three dogs receiving either placebo, SSBB or PTB treats were within normal ranges.

Slightly lower platelet counts in Dog 1 (SSBB) and Dog 2 (placebo) were considered to be related to a concurrent infection with *Ehrlichia canis* [14] which is endemic in Costa Rica [15]. In addition dog #1 developed slightly elevated globulin 3 days after the start of treatment which was also attributed to concurrent *Ehrlichia canis* infection. Prior to their admission at the shelter, the dogs were strays and therefore were likely exposed to infection with *Ehrlichia canis*, which may explain the elevated globulin and thrombocytopenia observed in these two dogs. Incubation time following infection with *Ehrlichia canis* can be few weeks and during that time animals may not show any signs of the disease. Despite mild thrombocytopenia and slightly elevated globulin the dogs did not show any clinical signs of illness, nor did they show signs of prolonged bleeding during sampling (Table 2).

Table 2. Summary of adverse events observed during the study

Treatment group	Test subjects	Treatment duration	Adverse events
SSBB (7x)	1	28 days	Transient thrombocytopenia from Day 3 to Day 14, no clinical signs of illness.
PTB (8x)	1	28 days	None
Placebo	1	28 days	Transient thrombocytopenia from Day 3 to Day 14, elevated globulin, no clinical signs of illness.

*SSBB = *Souroubea* spp Botanical Blend; PTB = *Platanus* Tree Bark

AHI, SSBB and PTB were orally administered to dogs at doses seven and eight times higher daily dose than the recommended daily dose; neither dose induced any systemic

or local adverse reactions which would be a safety concern for the dogs. Following the study the dogs were placed with new owners who have not reported any problems.

In conclusion, under the conditions of this study and according to the data from daily observations, physical examinations, body weight, hematology, biochemical and urinalysis both SSBB and PTB had no untoward effects on the health of dogs and were deemed safe.

The toxicology study with three dogs in Costa Rica served as a pilot screening for any negative health effects for each SSBB and PTB after prolonged and elevated administration. This study preceded the larger controlled target animal safety and toxicology trial (manuscript in preparation). Following the target safety study, an efficacy study with age variable populations of dogs was performed to evaluate Sin Susto™ as an anxiolytic product (manuscript in preparation). Furthermore, studies to evaluate the interaction of Sin Susto™ with commonly prescribed drugs and the withdrawal effects after chronic use of Sin Susto™ in dogs are underway.

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KLINIČKA ZAPAŽANJA I BEZBEDNOST DVA BILJNA PROIZVODA *SOUROUBEA* I *PLATANUS* SPP NAKON ORALNE PRIMENE; PRELIMINARNA TOKSIKOLOŠKA ISPITIVANJA KOD PASA

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Ova preliminarna studija procenjuje toksičnost i bezbednost dva biljna sastojka *Souroubea spp* Botanical Blend (SSBB) i Platanus Tree Bark (PTB) nakon oralne primene kod pasa u povišenim dozama u trajanju od 28 dana. Biljne mešavine SSBB i PTB su dva glavna aktivna sastojka u Sin Susto™, novog prirodnog proizvoda registrovanog za preventivu/smanjenje anksioznosti kod pasa. Tri zdrave ženke pasa su primale povišene doze SSBB, PTB ili placebo nakon čega je praćena pojava sistemskih ili lokalnih neželjenih efekata. Podaci iz ovog preliminarnog istraživanja su pokazali da SSBB i PTB nisu izazvali nikakve neželjene efekte kod pasa i mogu se smatrati bezbednim što je omogućilo dalje istraživanje i izvođenje kontrolisanih ispitivanja u cilju demonstriranja bezbednosti i toksičnosti preparata Sin Susto™ kod pasa.