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Review Article

Theme: Phytopharmaceuticals for Global Health: From Discovery to Approval
Guest Editor: Pramod B. Mahajan

New Botanical Anxiolytics for Use in Companion Animals and Humans

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Abstract. As part of our ongoing research into botanical therapies for anxiety disorders, the neotropical vine *Souroubea sympetala* was chosen for study as a phytochemical discovery strategy focusing on rare Central American plant families. When orally administered to male Sprague-Dawley rats, the crude plant extract, its ethyl acetate fraction, supercritical carbon dioxide fraction, or its isolated triterpenes reduced anxiety and/or fear-related behavior in standardized behavioral models. Pharmacological studies showed that the extracts acted at the benzodiazepine $GABA_A$ receptor and reduced corticosterone levels. A preparation containing *Souroubea* fortified with a second triterpene containing plant, *Platanus occidentalis*, was shown to be safe in a 28-day feeding trial with beagles at 5 times the intended dose. Subsequent trials with beagles in a thunderstorm model of noise aversion showed that the material reduced anxiety behaviors and cortisol levels in dogs. The formulation has been released for the companion animal market in Canada and the USA under the Trademark "Zentrol." Ongoing research is exploring the use of the material in treatment of anxiety and post-traumatic stress in humans.

KEYWORDS: anxiety; canine noise aversion; elevated plus maize; GABAA receptor; souroubea.

INTRODUCTION

During the last two decades, our labs at the University of Ottawa and Universidad Nacional have had an international collaboration to study, preserve, and utilize plant biodiversity for medicinal purposes, especially safe treatments for anxiety. As a discovery strategy, we focused on poorly studied plants, including the genus *Souroubea* (Fig. 1), which belongs to a small family of neotropical lianas and shrubs, the Marcgraviaceae, native to Central America, the northern part of South America, and the Caribbean Islands. Two species collected in Costa Rica, *Souroubea gilgii* Gilg. *and S. sympetala* V.A. Richt., were the most promising extracts studied for anxiety treatment in standardized rodent behavioral assays among extracts of many species surveyed and were selected for pharmaceutical development for animal and human health. The phytochemistry of the two species is very similar with triterpenes being the main bioactive molecules (Fig. 2) (1).

Anxiety disorders are among the most prevalent mental health problems in North America (2). Chronic use of conventional anxiolytic drugs is associated with adverse side effects, including sedation, cognitive impairment, impaired psychomotor coordination, and risk of tolerance, driving the need to find safer alternatives (3). There is a growing body of evidence which shows that complementary and alternative treatment approaches, including traditional herbal remedies, are increasingly used by anxiety patients (4–6). Although several botanical species show efficacy in mood disorders, two of the most effective botanicals, St John's Wort and Kava, have been associated with adverse effects and safe alternatives are needed.

Our discovery of anxiolytic effects of *Souroubea* extracts in rodents led to a subsequent investigation of traditional uses of *Souroubea* spp. for mental health. Studies by our research group showed that Q'eqchi' Maya healers in Belize used *Souroubea sympetala* for the treatment of witchcraft, the symptoms of

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Fig. 1. *Sououbea sympetala* vine in flower (left). Vine in plantation (right)

which include becoming withdrawn, showing little interest in daily life, and reduced verbal communication (1). Other ethnobotanical uses of this genus also point to its psychopharmacological properties. The use of *Souroubea guianensis* Aublet var. coralline (Mart) Wittmack by the Kubuyari in the Amazon as a calming agent for symptoms of nervousness in elders have been reported (7). *Souroubea guianensis* Aublet var. cylindrical Wittmack is used as a tranquilizing medicine by the Karijona and as a treatment for "susto" by the Taiwanos. *Susto*, a culture-specific syndrome, has been described as sickness which occurs in response to a frightening event. As well, previous investigations of plants used as remedies for the treatment of *susto* have demonstrated pharmacological activity at targets of anxiolytic drugs (8) and reduced anxiety-like behavior in rodents (9).

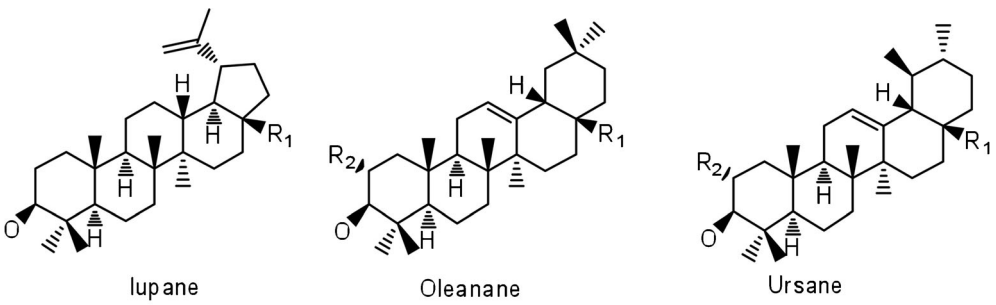
PRECLINICAL STUDIES IN RODENTS

A series of pre-clinical studies using orally administered *Souroubea* raw plant materials, crude ethanol and ethyl acetate extracts, or supercritical CO_2 extracts have demonstrated anxiolytic potential in rodents using standardized behavioral assays on short-term and long-term treatment regimens (1,10). Behavioral assays were conducted to elucidate the dose-response effects of *Souroubea* spp. ethanol extract in rats in the elevated plus maze (EPM) at 25, 50, 75, and 100 mg/kg (1). Treatments led to dose-dependent increases in the time spent in the open arms of the EPM, and unprotected head dips, both of which are measures of anxiolytic activity (Fig. 3). The extract was also demonstrated to significantly reduce the time spent freezing in the conditioned emotional response (CER) test (12). Different extraction methods (10) showed that supercritical extracts were the most effective with significant effects at 25 mg/kg. Leaf was more effective than stem, and there was little difference between the two species studied (*S. gilgii* and *S. sympetala*), a result consistent with their similar phytochemistry.

Bioassay-guided fractionation experiments with sequential hexane, ethyl acetate, and water fractions showed that the active principles were enriched in the ethyl acetate fraction. The orally administered ethyl acetate fraction was found to significantly increase time spent in the open arms by rats in the EPM at 50 and 100 mg/kg body weight (11,12). Similarly, at 75 or 100 mg/kg, this fraction reduced freezing time in the contextual CER test (13) and attenuated expression of fear-potentiated startle (FPS) (12). The fraction also significantly increased total active social interaction at 100 mg/kg (12). Interestingly, anxiolytic activity of the crude extract in the FPS test was significantly greater than the active ethyl acetate fraction, suggesting that there may be pleiotropic effects at play (12).

PHYTOCHEMCIAL CONSTITUENTS, ACTIVE PRINCIPLES, AND ANALOGS

Five triterpenes (Fig. 2) were isolated from the active ethyl acetate fraction. Of these, betulinic acid (BA), a lupane-type triterpene, induced anxiolytic-like activity upon oral administration at 1 mg/kg in the EPM (Fig. 4) (11). Administration of 0.5 mg/kg of BA increased the percentage of time spent in the open arms and the number of unprotected head dips, an indication of increased risk



Triterpene family	Triterpene	R1	R2	Molecular Weight (g/mol)
Lupane	Betulinic Acid	COOH	---	456.71
	Lupeol	CH_3	---	426.70
Oleanane	β-Amyrin	CH_3	H	426.70
Ursane	α-Amyrin	CH_3	---	426.70
	Ursolic acid	COOH	---	456.71

Fig. 2. Major triterpenes identified in *Souroubea* spp. Betulinic acid is the main anxiolytic principle and its activity is enhanced by amyrins. Lupeol and usroslic acid are inactive at doses used in rodent models

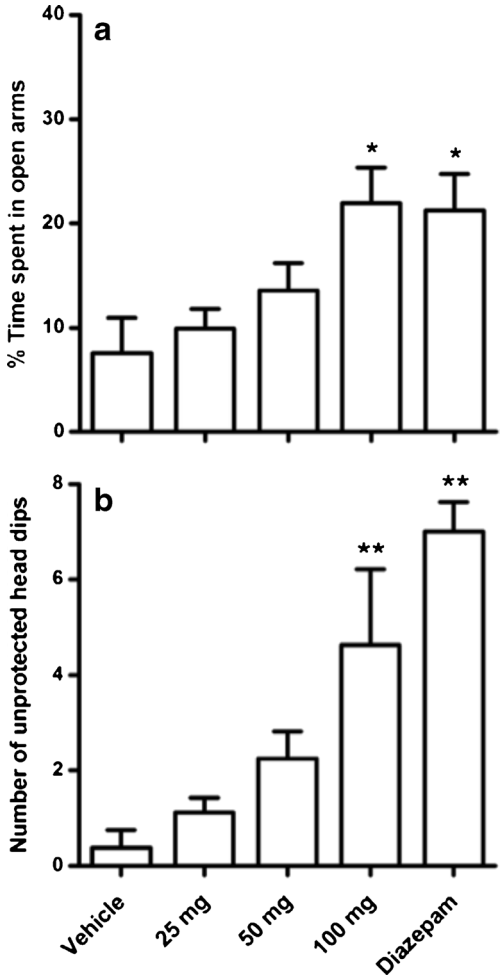


Fig. 3. Effects of diazepam (2 mg/kg) and dose response in mg/kg of the crude ethanolic extract of leaves of *S. sympetala* on the percentage of time spent on the open arms (**a**) and the number of unprotected head dips (**b**) in the elevated plus maze by orally administered rats (n = 8 for vehicle, n = 8 for all crude ethanolic extract doses, n = 12 for diazepam). $p < 0.005$ and $p < 0.001$ indicate significant differences from vehicle. Reprinted from with permission, from Elsevier © 2015: Puniani E, Cayer C, Kent P, Mullally M, Sánchez-Vindas P, Poveda Álvarez L, Cal V, Merali Z, Arnason JT, Durst T. Ethnopharmacology of *Souroubea sympetala* and *Souroubea gilgii* (Marcgraviaceae) and identification of betulinic acid as an anxiolytic principle. Phytochemistry 2015;113:73–78

assessment behavior, and significantly decreased the FPS response compared to the vehicle controls (12).

These findings were bolstered by parallel studies in different strains of mice, including a genetically anxious Balb/c strain, a model of trait anxiety. Intraperitoneal BA administered at 0.25 mg/kg markedly reduced anxiety-like behavior in these mice. Betulinic acid had been previously shown to be pharmacologically active with anti-melanoma and anti-HIV properties (14).

Other compounds present in *Souroubea* spp., including α- and β-amyrins and ursolic acid, were not found to be active in the EPM at the same dose as betulinic acid (1). However, anxiolytic and antidepressant effects have been demonstrated for α- and β-amyrins by others (15). In behavioral assays, the combination of amyrins and betulinic acid was synergistic in promoting anxiolytic effects.

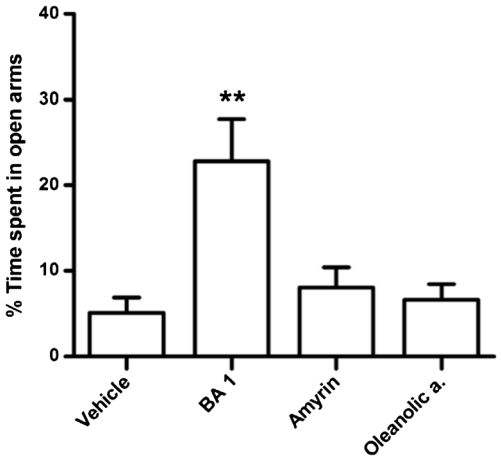


Fig. 4. Effects of several structurally related compounds: betulinic acid 1 (0.5 mg/kg), α- and β-amyrins (0.5 mg/kg), and oleanolic acid (0.5 mg/kg) on the percentage of time spent on the open arms in the elevated plus maze by orally administered rats (n = 10 for all groups). $p < 0.01$ indicates a significant difference from vehicle. Reprinted with permission from Elsevier © 2015: Puniani E, Cayer C, Kent P, Mullally M, Sánchez-Vindas P, Poveda Álvarez L, Cal V, Merali Z, Arnason JT, Durst T. Ethnopharmacology of *Souroubea sympetala* and *Souroubea gilgii* (Marcgraviaceae) and identification of betulinic acid as an anxiolytic principle. Phytochemistry 2015;113:73–78

Analogs of BA, particularly the methyl ester derivative, have shown comparable or higher activity compared to BA in the social interaction (SI) test when tested at 0.5 mg/kg upon i.p. and oral administration (11) and in the EPM at 5 mg/kg when orally delivered (16). The active principles were isolated by bioassay-guided isolation and identified as triterpenes by comparison of their spectral data with literature values (1).

PHARMACOLOGY OF SOUROUBEA SPP.

In addition to behavioral modification, we have demonstrated that *Souroubea* spp. extracts induce physiological changes at targets of anxiety and stress. Evidence of anxiolytic activity of *Souroubea* spp. in unconditioned (EPM, SI) and conditioned (CER, FPS) tests suggested that *Souroubea* extracts and (or) compounds may be modulating anxiety responses by acting as agonists at the $GABA_A$-BZD receptor (17–19). Mullally *et al.* (16) showed that in the presence of a $GABA_A$-BZD receptor-specific antagonist, flumazenil, the anxiolytic activity of extracts of *Souroubea* as well as methyl-BA were markedly attenuated in the EPM, and to a lesser degree, in the CER (Fig. 5).

Investigations (13) in *Onchorhyncus mykiss* (rainbow trout) showed that BA was active at the interrenal cells of the hypothalamic-pituitary-interrenal axis in lowering cortisol production (Fig. 6). Upon *in vivo* administration at 1 mg/kg in acutely stressed fish, BA lowered plasma cortisol but had no effect on plasma cortisol levels in unstressed controls. These cortisol-lowering effects in stressed animals have been observed (20) in other species including rats (corticosterone), piglets, dogs, and horses.

Additional mechanisms may contribute to the observed anxiolytic effects that have yet to be tested. Treatment with *Souroubea sympetala* (supercritical CO_2 extract) has been

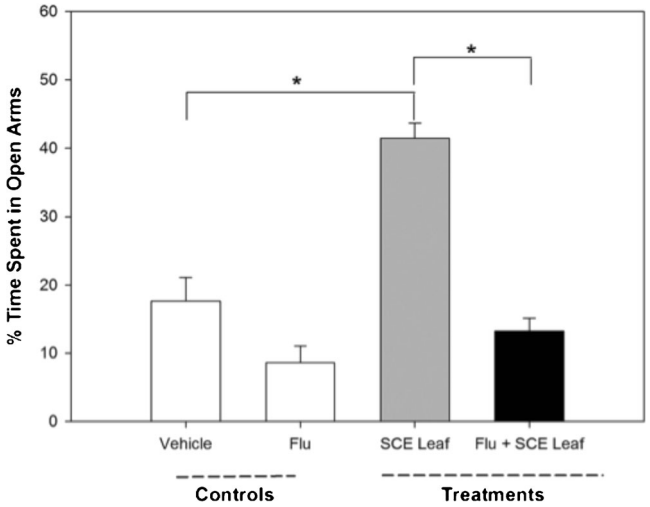


Fig. 5. Effect of pretreatment with flumazenil on percent total time spent freezing in the conditioned emotional response (context) assay. All values represent the group mean ± SEM of n = 7–16 per group. Vehicle, vehicle control (50% sweetened, condensed milk); Flu, flumazenil, i.p. (0.5 mg/kg), EtOAc leaf, ethyl acetate extract of *S. sympetala* leaf (75 mg/kg); Flu + EtOAc leaf, flumazenil (0.5 mg/kg) followed by ethyl acetate extract of *S. sympetala* leaf (75 mg/kg) 45 min later. Asterisk indicates significant difference from vehicle control, one-way ANOVA, with Dunnett's *post hoc* test $p < 0.05$. © 2008 Canadian Science Publishing or its licensors. Reproduced with permission from Mullally M, Cayer C, Kramp K, Otárola Rojas M, Sanchez Vindas P, Garcia M, Poveda Alvarez L, Durst T, Merali Z, Trudeau VL, *et al. Souroubea sympetala* (Marcgraviaceae): a medicinal plant that exerts anxiolysis through interaction with the $GABA_A$ benzodiazepine receptor. Can. J. Physiol. Pharmacol. 2014; 92:758–64

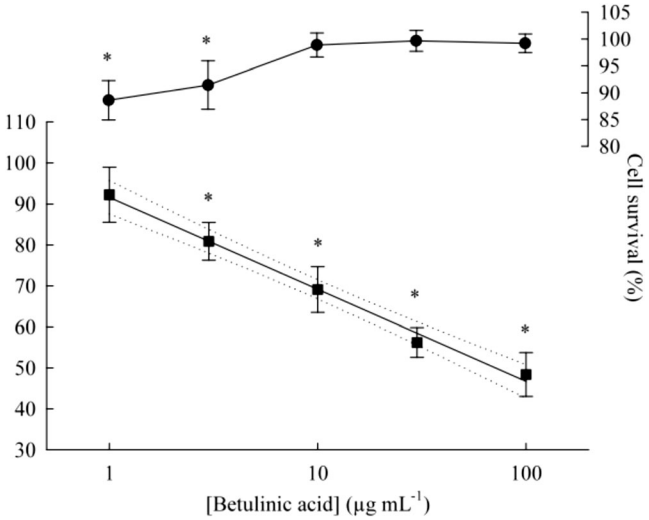


Fig. 6. Effect of incubation with betulinic acid (BA) on ACTH-stimulated cortisol production by rainbow trout (*Oncorhynchus mykiss*) head kidney preparations. Square symbols represent ACTH-stimulated cortisol production as a percentage of the positive control (ACTH-stimulated cortisol production in the absence of BA) whereas circles indicate cell viability at each extract concentration as a percentage of the positive control (viability of ACTH-stimulated preparations in the absence of BA). Values represent means ± SEM, N = 7 independent preparations; the dotted lines represent the 95% confidence interval, and the linear regression equation was cortisol production (%) = 91.65–22.46 log (BA concentration), R^2 = 0.587, $P < 0.001$. An asterisk indicates a value that is significantly different from the positive control (one-way RM ANOVA, P = 0.005 for cell viability and < 0.001 for cortisol production). Reprinted with permission from Elsevier © 2017 from, Mullally M, Mimeault C, Rojas M, Vindas P, Garcia M, Alvarez L, Moon TW, Gilmour KM, Trudeau V, Arnason JT. A botanical extract of *Souroubea sympetala* and its active principle, betulinic acid, attenuate the cortisol response to a stressor in rainbow trout, *Oncorhynchus mykiss.* Aquaculture. 2017;468 (1): 26–31

shown to increase the number of unprotected head dips in the EPM, which is a behavioral parameter responsive to 5HT1A (serotonin receptor 1A) ligands (10). Interestingly, response in the SI test appears to also involve serotoninergic receptors (20) and there are reported reciprocal interactions between GABA and serotonin circuitry (21,22). Some recent data showed fatty acid amide hydrolase inhibition, implicating the endocannabinoid system.

DEVELOPMENT OF A LOW RISK VETERINARY PRODUCT

In 2010, we initiated a commercial product for veterinary applications as a canine calming agent for noise aversion in collaboration with Bioniche Animal Sciences. To provide a cost-effective product, *Souroubea* spp. vine was fortified (55:45 *w*/*w*) with sycamore bark (*Platanus occidentalis*), a native North American botanical which contains large amounts of betulinic acid (BA) but no amyrins (23). Studies of the two raw (unextracted) botanicals, individually or as part of a blend, were undertaken in rodent models and demonstrated that the latter was more active than the individual components (Fig. 7). The raw botanical ingredients were formulated for dogs in a 3.5 g chewable tablet containing binder and beef flavoring and 2 g of a 55:45 mixture of *Souroubea* spp. and *Plantanus* spp. standardized to provide the recommended dose of 1 mg/kg betulinic acid for a 10 kg dog. The individual plant components and blend were well tolerated in a pilot dog study in a sub-acute (28 days) pilot study at 8 times recommended dose (24). Safety was confirmed (25) in a full scale independent toxicology trial at 5 times dose while monitoring weight, clinical chemistry, and hematology in beagles by an independent study at Kingfisher Inc. with no significant adverse effects. Studies in dogs at Cancog Technologies in Guleph, Ontario using a thunderstorm model of noise aversion indicated significant dose-dependent anxiolytic effects through behavioral modification as well as dose-dependent reduction in plasma cortisol (26). A subsequent blinded placebo-controlled study indicated that administration of a single dose of the blend was effective at significantly ($p < 0.05$) lowering cortisol levels in treated animals compared to placebo, 1 h after administration.

In 2013–2014, Bioniche sold its veterinary products division. The University of Ottawa license for the technology was then transferred to Souroubea Botanicals Inc. (Ottawa ON), who have marketed the canine tablet through a US distributor (Zentrol™) since June 2016.

SUSTAINABLE PRODUCTION

Widespread traditional use or commercialization of wild harvested botanicals can lead to their extirpation. North

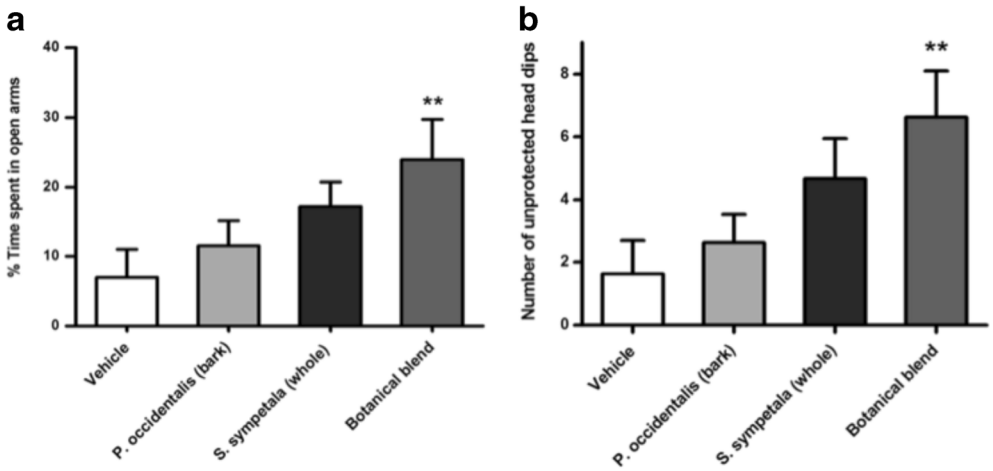


Fig. 7. Effects of raw plant materials on the **a** percentage of time spent in the open arms and **b** number of unprotected head dips in rats in the elevated plus maze following 3-day oral administrations of their respective treatments (100 mg/kg for *P. occidentalis*, 1000 mg/kg for *S. sympetala*, 200 mg/kg for the botanical blend). $^{**}P < 0.01$ indicates a significant difference from vehicle

American ginseng and African Pygeum are two well-known examples of botanicals that were severely depleted by unregulated collection. Sustainable production of biodiversity sources is best achieved by cultivation. When we first attempted propagation of *Souroubea symeptala* or *S. gilgii* from cuttings using a variety of treatments, we observed 70–100% mortality. Seeds were recalcitrant and had a germination capacity of only 1–2%. The situation was first resolved by use of air layering to produce 5–10 viable rooted plants for a vine. In addition, the use of *in vitro* culture techniques at Universidad Nacional researchers improved seed germination to 90%. The first *Souroubea sympetala* and *S. gilgii* plantation was produced in Sarapiqui, Costa Rica in 2014–2016 by UNA researchers, and plants have grown into vigorous bushes over 2 m high (Fig. 1). Sustainable harvests of over 1 ton/ha were achieved every 6 months, followed by vigorous regrowth.

Sustainable harvest of *Platanus occidentalis* bark can be made using wild crafting from trees in Eastern North America, as the bark sheds without damage to the tree in late summer. Up to 10 kg/h can be harvested by an experienced collector. Production of either of these species provides opportunities for local indigenous communities.

STANDARDIZATION STABILITY AND PHAMACOKINETICS

One of the barriers to the availability of evidence-based botanical products is lack of standardization which prevents comparison of the clinically studied products with marketed products. We have developed an HPLC-APCI-MS method for targeted phytochemical analysis of active principles, including BA and other triterpenes such as ursolic acid, α- and β-amyrins, and lupeol, which has been updated (27) and validated for both species. The triterpenes present in *Souroubea* spp. (amyrins, lupeol, BA, urosolic acid, 2-hydroxyursolic acid) and in *Platanus* spp. (BA and platanic acid) are thermally very stable. Samples stored at room temperature for more than 1 year showed no signs of degradation, either by visual inspection or when investigated analytically.

Pharmacokinetics of BA has been studied by other researchers. Even when administered at very high doses, it appears in the serum in very low concentrations and accumulates in fatty organs, e.g., the liver and the brain (28). In dogs administered an anxiolytic 1 mg/kg dose, BA and its predicted metabolites were below detection limits in serum (25), but significant cortisol lowering occurred. Thus, instead of monitoring serum BA, cortisol is a better biomarker of efficacy.

ENTRY INTO THE HUMAN MARKET

Based on preliminary studies in rats and dogs, *Souroubea/Platanus* botanical extract is a promising candidate to study for managing anxiety symptoms in humans. Unlike conventional anxiolytics, long-term administration of *Souroubea* active principles does not lead to tolerance or withdrawal effects in rodents. In a study by our group (12), administration of methyl-BA over 30 days resulted in significantly higher percent time spent in the open arms as well as number of unprotected head dips in the EPM compared to controls, i.e., potency and efficacy was sustained over repeated administration. Withdrawal effects of BA and methyl-BA-treated animals assessed during the light and dark cycles using behavioral parameters, food intake, fecal boli output, and body weights were not significantly different from controls (12). There is no clinical data on *Souroubea/Platanus* drug interactions, but we have conducted *in vitro* tests which indicate low potential of inhibition of key cytochrome P450 enzymes, CYP 3A4 and 2D6, which are implicated in many drug-drug interactions (unpublished). As well, addition of *Souroubea/Platanus* extract had little effect on valium metabolism in human liver microsomes (unpublished).

The Canadian Natural Health Product regulatory framework provides an opportunity for licensing *Souroubea/Platanus* extract as an approved low-risk product based on evidence of safety, quality, and efficacy with a limited treatment claim. Approvals for clinical trials with a *Souroubea/Platanus* extract investigational product (95% ethanol extract) are currently under consideration. Subsequently, the product could be marketed as a US dietary

supplement with a structure-function statement (a statement that describes the role of a dietary ingredient in humans without making a treatment claim). Recent experiments in rodents examined the effects on auditory fear memory reconsolidation elicited by the extract. In the fear-potentiated startle paradigm, significant impairment of long-term fear memory retention was observed, and no significant effects on short-term memory occurred when the botanical was administered orally directly following memory reactivation. This suggests the potential application in post-traumatic stress disorder in humans. If this can be demonstrated clinically, a standardized extract is a candidate for registration in the US as a botanical drug with evidence-based anxiolytic claims.

ACKNOWLEDGEMENTS

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest Several of the authors, Durst, Merali, Sanchez, Arnason, Baker, and Masic have direct involvement in Souroubea Botanicals Inc. which has brought the research to market in animal care field.

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