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The effect of *Salvia divinorum* and *Mitragyna speciosa* extracts, fraction and major constituents on place aversion and place preference in rats

Kenneth J. Sufka [a,b,d,*], Melissa J. Loria [a], Kevin Lewellyn [c], Jordan K. Zjawiony [c,d], Zulfiqar Ali [e], Naohito Abe [e], Ikhlas A. Khan [c,d,e]

[a] Department of Psychology, University of Mississippi, University, MS 38677, USA
[b] Department of Pharmacology, University of Mississippi, University, MS 38677, USA
[c] Department of Pharmacognosy, University of Mississippi, University, MS 38677, USA
[d] Research Institute of Pharmaceutical Science, University of Mississippi, University, MS 38677, USA
[e] National Center for Natural Product Research, University of Mississippi, University, MS 38677, USA

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ABSTRACT

Ethnopharmacological relevance: Consumer use of botanicals has increased despite, in many instances, the paucity of research demonstrating efficacy or identifying liabilities. This research employed the place preference/aversion paradigm to characterize the psychoactive properties of *Salvia divinorum* extract (10, 30, 100 mg/kg), salvinorin A (0.1, 0.3, 1.0 mg/kg), *Mitragyna speciosa* MeOH extract (50, 100, 300 mg/kg), *Mitragyna speciosa* alkaloid-enriched fraction (12.5, 25, 75 mg/kg) and mitragynine (5, 10, 30 mg/kg) in rats.

Material and methods: Following apparatus habituation and baseline preference scores, male Sprague-Dawley rats were given eight counter-balanced drug versus vehicle conditioning trials followed by a preference test conducted under drug-free states. *S*(+)-amphetamine (1 mg/kg) served as the positive control (in Exp. 2) and haloperidol (0.8, 1.0 mg/kg) served as the negative control in both studies.

Results: Rats displayed place aversion to both *Salvia divinorum* and salvinorin A that exceeded that of haloperidol. Rats showed place preference to mitragynine that was similar to that of *S*(+)-amphetamine. This CPP effect was much less pronounced with the *Mitragyna speciosa* extract and its fraction.

Conclusions: These findings suggest that both botanicals possess liabilities, albeit somewhat different, that warrant caution in their use.

1. Introduction

Numerous botanical products are widely available to consumers and used not only to treat various medical conditions but also, in some instances, for their pleasurable/euphoric properties (Dennehy et al., 2005). Botanical products give consumers the impression that since they are "all natural" they are safe and pose no physical or psychological health risks (Marcus and Grollman, 2002. Whereas botanicals used therapeutically often have some supportive evidence of their efficacy, those used recreationally are typically under-researched. Such recreational botanicals contain a wide array of constituents whose properties, such as toxicity and abuse potential, may make them potentially dangerous. One recent example of this is the hepatotoxic effect of the purported anxiolytic kava–kava (Humberston et al., 2003; Teschke et al., 2008). Research to characterize the putative liabilities of botanicals and their constituents are as necessary as research that establishes claims of therapeutic efficacy.

One procedure to evaluate a compound's abuse potential is the Conditioned Place Preference (CPP) procedure. This associative learning paradigm is based on the notion that animals prefer environments previously paired with positively reinforcing drugs (Bardo and Bevins, 2000). It should be noted that compounds that possess unpleasant properties produce conditioned place aversion (CPA) in the paradigm. CPP/CPA, in its traditional use of studying single-entity compounds, may not lend itself well to studying complex botanical products. Botanicals possess a wide range of constituents that may have antagonistic or synergistic effects that mask or exacerbate liabilities, respectively. Thus, studying only a major constituent or the entire extract alone may fail to identify potential liabilities. One approach is to concomitantly evaluate the full extract, one or more of its fractions and its major constituent(s) in the paradigm. This strategy would reveal antagonistic or synergistic effects within the extract and fraction and more fully characterize constituent liabilities.

* Corresponding author at: Peabody Building, University of Mississippi, University, MS 38677, USA. Tel.: +1 662 915 7728.
E-mail address: pysufka@olemiss.edu (K.J. Sufka).

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For the present research, we used this extract-fraction-constituent strategy to study the liabilities of the widely available *Salvia divinorum* (*Salvia divinorum)* and *Mitragyna speciosa* (*Mitragyna speciosa*) both of which are used recreationally. *Salvia divinorum* and its major active metabolite salvinorin A possess hallucinogenic properties. Salvinorin A is a κ-opioid receptor (KOR) agonist (Roth et al., 2002; Chavkin et al., 2004) and KOR agonists, including salvinorin A, are reported to produce unpleasant effects in humans and, not surprisingly, cause CPA in rodent models (Mucha and Herz, 1985; Pfeiffer et al., 1986; Zhang et al., 2005). *Mitragyna speciosa* and its major active alkaloid mitragynine possess stimulant like effects at low doses and opiate-like effects at higher doses (US-DOJ). Mitragynine is a high affinity μ-opioid receptor (MOR) agonist (Watanabe et al., 1997; Yamamoto et al., 1999) and the role MOR in addiction is well-documented (for review see Koob et al., 1998). In rodents, μ-opioid receptor agonists produce CPP (Tzschentke, 2007). We expect salvinorin A and mitragynine to produce CPA and CPP, respectively. Whether *Salvia divinorum* and *Mitragyna speciosa* contain other psychoactive constituents that mask or exacerbate the effects of their major metabolites is unknown.

2. Materials and methods

2.1. Subjects

The research protocols detailed below were approved on 12 June 2012 by the university's IACUC (protocol # 12–020). Male Sprague Dawley rats (175–200 g, 6–7 weeks old; Harlan, Indianapolis, IN) were housed in pairs and maintained under a 12-h light/dark cycle in a temperature and humidity controlled vivarium. Food and water were available ad libitum. Animals were handled daily (3 d) prior to experimental manipulations to reduce experimenter-related stress.

2.2. Apparatus and procedure

Five place preference chambers (Model MED CPP RS; Med Associates, St. Albans, VT) were used for these experiments. Each chamber has two stimulus-distinct (black versus white colored walls and wire mesh or metal rod flooring) drug-conditioning chambers and a third central start chamber (colored gray with smooth solid surface floor). Guillotine doors provide confinement/access to the conditioning chambers. The CPP/CPA procedure involves four phases: (1) a 15 m apparatus habituation trial, (2) a 15 m baseline preference trial, (3) eight 30 m drug conditioning trials, and (4) a final 15 m place preference trial. Animals had access to the entire place preference apparatus during the drug-free habituation, baseline preference and final preference trials. The conditioning phase involved alternate day, counterbalanced (for drug order) pairings of test compound in one compartment (S+) and vehicle in the other (S−). Conditioning trials were counter-balanced (drug/vehicle) within treatments conditions. Assignment of test compound to a given compartment (S+) was based on baseline preference scores where compounds expected to produce CPP and CPA were assigned to the non-preferred and preferred compartments, respectively. Test apparatus was thoroughly cleaned after each trial.

2.3. Test compounds

The leaves of *Salvia divinorum* and *Mitragyna speciosa* were purchased from the *Salvia divinorum* Research Center (Malibu, CA USA) and Bouncing Bear Botanicals (Lawrence, KS USA), respectively. The plant material was identified by Dr. Vijayasankar Raman at The National Center for Natural Prooducts Research at the University of Mississipp (Oxford, MS USA). *Salvia divinorum* voucher no. 13458 and *Mitragyna speciosa* voucher no. 12433.

Salvia divinorum extract (10, 30, 100 mg/kg) was prepared by exhaustive extraction of dry plant material with ethanol. The extract was filtered and then concentrated. Because it is well-established that the psychoactive properties of *Salvia divinorum* are mediated by salvinorin A, we opted to not include analysis of a *Salvia divinorum* fraction. Salvinorin A (0.1, 0.3, 1.0 mg/kg) was isolated from *Salvia divinorum* leaves as previously described (Munro and Rizzacasa, 2003). Briefly, *Salvia divinorum* leaves were extracted with acetone and subsequently recrystallized from 95% ethanol to yield 99% (HPLC) pure salvinorin A. The salvinorin A doses selected were based on previously published studies in rodent models (McCurdy et al., 2006) and, for *Salvia divinorum*, dosing equivalence based on concentrations of salvinorin A in the extract. The HPLC fingerprinting analysis of *Salvia divinorum* extract showed that salvinorin A existed as one of the major constituents of *Salvia divinorum* (3.1%; see Supplemental materials. In this study, haloperidol (1 mg/kg, Sigma-Aldrich Inc., >98% purity) served as the negative control for CPA. Two vehicles were employed in this study ($n=5$). For *Salvia divinorum* and salvinorin A the vehicle was 10% DMSO and 10% Tween80 in saline. For haloperidol the vehicle was 50% DMSO in saline. ANOVA in vehicle groups did not reveal significant differences on preferences scores and these groups were combined for subsequent analyses.

The leaves of *Mitragyna speciosa* (550 g) were extracted with methanol (3 L) for 24 h at room temperature for four times. The solvent was removed under reduced pressure to yield a dried extract. An aliquot was suspended in 5% HCl in water and extracted with ethyl acetate. The water-soluble part was basified (pH 9–10) with liquid ammonia and extracted with ethyl acetate. The ethyl acetate-soluble part, separated from basic media, was dried under reduced pressure to get an alkaloid-enriched fraction. Mitragynine (97% pure) was isolated from the fraction by repeated column chromatogaraphy over silica gel using chloroform/methanol (9:1) and hexanes/acetone/liq. ammonia (210:90:1) solvent systems (see Supplemental materials). From these processes, *Mitragyna speciosa* extract (50, 100, 300 mg/kg), an alkaloid-enriched fraction (12.5, 25, 75 mg/kg) and mitragynine (5, 10 and 30 mg/kg) were used in this study. The mitragynine doses selected were based on previously published studies in rodent models (Sabetghadam et al., 2013) and for *Mitragyna speciosa*, the dosing was based to maximize concentrations of mitragynine in the extract and fraction (approximately 0.5, 1.0, 3.0 mg/kg of mitragynine, respectively) but to avoid its preparation as a suspension. Mitragynine was found to be the major compound in the crude extract (3.5%) and alkaloidal enrich fraction (4.3%) during HPLC fingerprinting analysis (see Supplemental materials). In this study, *S*(+)-amphetamine (1 mg/kg in saline Sigma-Aldrich Inc., >99% purity) served as the positive control for CPP and haloperidol (0.8 mg/kg) served as the negative control for CPA. As before, two vehicles ($n=5$) were employed in this study. *Mitragyna speciosa* extracts, fractions and constituents were dissolved 20% Tween80 in saline. The haloperidol vehicle was 50% DMSO in saline. ANOVA in vehicle groups did not reveal significant differences on preferences scores and these groups were combined for all subsequent analyses. All test compounds or vehicles were administered via intraperitoneal (IP) injection in a volume of 1 mL/kg immediately before each conditioning trial. Sample sizes were $n=10$.

2.4. Statistics

Data acquisition was handled by infrared photo-beam detection via MED-PC® IV software. Data analyses were conducted using SPSS® software. Group differences (CPP or CPA) were analyzed using one-way ANOVAs. CPP/CPA scores were defined as time in