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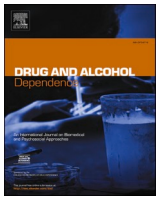

Vaporized D-limonene selectively mitigates the acute anxiogenic effects of Δ9-tetrahydrocannabinol in healthy adults who intermittently use cannabis

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ABSTRACT

Background: Cannabis contains hundreds of chemical constituents beyond delta-9-tetrahydrocannabinol (THC), which is believed to drive most of its acute pharmacodynamic effects. The entourage effect theory asserts that non-THC constituents can impact acute cannabis effects, but few empirical studies have systematically evaluated this theory in humans. This study assessed whether the cannabis terpenoid D-limonene mitigates the acute anxiogenic effects of THC.

Methods: Twenty healthy adults completed nine, double-blind outpatient sessions in which they inhaled vaporized THC alone (15 mg or 30 mg), D-limonene alone (1 mg or 5 mg), the same doses of THC and D-limonene together, or placebo; a subset of participants (n=12) completed a tenth session in which 30 mg THC+15 mg D-limonene was administered. Outcomes included subjective drug effects, cognitive/psychomotor performance, vital signs, and plasma THC and D-limonene concentrations.

Results: When d-limonene was administered alone, pharmacodynamic outcomes did not differ from placebo. Administration of 15 mg and 30 mg THC alone produced subjective, cognitive, and physiological effects typical of acute cannabis exposure. Ratings of anxiety-like subjective effects qualitatively decreased as D-limonene dose increased and concurrent administration of 30 mg THC+15 mg D-limonene significantly reduced ratings of "anxious/nervous" and "paranoid" compared with 30 mg THC alone. Other pharmacodynamic effects were unchanged by D-limonene. D-limonene plasma concentrations were dose orderly, and concurrent administration of D-limonene did not alter THC pharmacokinetics.

Conclusions: D-limonene selectively attenuated THC-induced anxiogenic effects, suggesting this terpenoid could increase the therapeutic index of THC. Future research should determine whether this effect extends to oral dose formulations and evaluate the interactions between other cannabis terpenoids or cannabinoids and THC.

1. Introduction

Cannabis is one of the most commonly used drugs in the world, and the prevalence of use is increasing as legalization of the drug expands for medicinal and non-medicinal purposes (Schulenberg et al., 2021; SAMHSA., 2020). Cannabis is often considered synonymous with delta-9-tetrahydrocannabinol (THC), the primary psychoactive constituent of the plant that is responsible for producing many of its hallmark effects. Specifically, THC is a partial agonist of cannabinoid type 1 (CB_1) and type 2 (CB_2) receptors and can produce both positive (e.g., feelings of euphoria, relaxed mood) and negative (e.g., acute anxiety and paranoia, cognitive impairment) effects when acutely administered (Newmeyer et al., 2017; Pertwee, 2008; Sharpe et al., 2020; Spindle et al., 2018). Cannabis plants have been selectively bred over time to

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contain greater concentrations of THC, and it is now common for cannabis flower sold in dispensaries to contain upwards of 20–30% THC (Cash et al., 2020; Freeman et al., 2021; Vergara et al., 2017). Beyond THC, the cannabis plant contains hundreds of additional constituents, including cannabidiol or CBD, so-called "minor" cannabinoids (e.g., cannabigerol or CBG, THCV, etc.), and terpenoids or "terpenes" (e.g., D-limonene, pinene, beta-caryophyllene; Hazekamp et al., 2016; Vergara et al., 2017; Russo and Marcu, 2017).

Historically, THC was believed to account wholly for the acute behavioral and psychoactive effects of cannabis and other cannabis constituents were considered largely inconsequential (Wachtel et al., 2002). However, an alternative view, commonly referred to as the cannabis entourage effect theory, asserts that many constituents of the plant (e.g., minor cannabinoids and/or terpenes) meaningfully influence the acute effects of cannabis through either unique pharmacological mechanisms or mechanisms that modulate the effects of THC (Russo, 2011). Though largely untested in empirical clinical research, the cannabis entourage effect theory has greatly influenced cannabis industry practices, including how cannabis products are cultivated, marketed, and consumed (Cogan, 2020). For example, cannabis is often selectively bred to contain specific minor cannabinoid and/or terpene profiles and there is a growing market of products purported to principally contain minor cannabinoids or terpenes (Cogan, 2020). Moreover, marketing materials for cannabis products, including advertisements and product packaging, often highlight minor cannabinoid and/or terpene profiles and may overtly state that the effects a person can expect to feel will differ based on these profiles (Caputi, 2022; Cogan, 2020; Luc et al., 2020). However, most of the claims made that relate to specific cannabis "entourage" interactions are largely theoretical and have not been empirically tested in controlled human studies.

To date, clinical research on interactions between THC and non-THC constituents has focused primarily on CBD, an abundant cannabinoid found in cannabis that, alone, typically does not produce acute intoxicating or impairing effects (Sholler et al., 2020). Research on THC-CBD interactions has been mixed, with some studies showing that CBD exacerbates THC's effects (Arkell et al., 2020; Bansal et al., 2023; Zamarripa et al., 2023), some showing that CBD mitigates THC's effects (Englund et al., 2013; Zuardi et al., 1982), and others showing no modulatory effects of CBD on THC (Haney et al., 2016; Ilan et al., 2005). The conflicting results across studies are likely due to methodological differences, including THC/CBD doses, timing of drug dosing, and route of administration. Beyond CBD, there are various other cannabis constituents believed to influence the effects of cannabis that remain understudied, including the terpene D-limonene (Russo, 2011).

D-limonene is one of the most abundant terpenes in the cannabis plant, though concentrations may vary widely across chemovars, and is ubiquitous in citrus fruits (e.g., lemons; Noma and Asakawa., 2010; Russo, 2011). Studies suggest that D-limonene has anxiolytic (i.e., anxiety-reducing) properties. For example, preclinical studies have demonstrated that D-limonene or citrus essential oils high in D-limonene reduce anxiety-like behavior in rodents, as evidenced by behavioral paradigms such the elevated plus maze and open field task (Buchbauer et al., 1993; Carvalho-Freitas and Costa, 2002; Komiya et al., 2006; Song et al., 2021). These findings have been replicated in a couple small clinical studies. For example, in one study, hospital patients undergoing a stressful bone marrow procedure were exposed in ambient air to a D-limonene-dominant essential oil or placebo (a saline solution), or an oral dose of diazepam; D-limonene lowered self-reported anxiety on the State-Trait Anxiety Inventory (STAI-S) and reduced blood pressure and heart rate (HR) relative to baseline, while the placebo did not elicit any of these effects and diazepam only reduced blood pressure (Pimenta et al., 2016). In a second clinical study that was not placebo-controlled, individuals hospitalized with depression were exposed in ambient air to a citrus fragrance containing predominantly D-limonene and reductions in depression on the Hamilton Rating Scale for Depression (HRSD) were observed, along with reductions in urinary cortisol and dopamine, and normalization of biomarkers of immune function (Komori et al., 1995). Though these prior studies collectively suggest that limonene may possess anxiolytic properties, controlled research to understand whether D-limonene may alter THC-induced anxiety or other pharmacodynamic effects is lacking.

Pharmaceutical formulations of THC (dronabinol) or the THC analog nabilone are widely approved for treating chemotherapy-induced nausea or to stimulate appetite in certain clinical populations (e.g., individuals with advanced HIV/AIDS; Beal et al., 1995, 1997). However, use of these medications is limited, in part, due to a narrow therapeutic index (i.e., an effective therapeutic dose is close to a dose that may elicit an adverse event; D'Souza et al., 2004; Favrat et al., 2005). As noted above, one of the most common adverse effects associated with THC or THC-dominant cannabis is acute anxiety or paranoid/panicked reactions (Freeman et al., 2015); these reactions are mediated via modulation of CB_1 receptors in the amygdala by THC following cannabis use (Bhattacharyya et al., 2017). Thus, the development of novel THC-based medications that mitigate the anxiogenic effects of THC, hence widening its therapeutic index, could be of considerable clinical benefit. Rigorous controlled clinical studies are necessary to examine whether alternative cannabis constituents aside from CBD, such as D-limonene, may increase the tolerability of THC.

Given D-limonene's purported anxiolytic properties, the primary aim of the present controlled human laboratory study was to examine whether D-limonene acutely mitigates the anxiogenic effects of THC, as hypothesized previously (Russo, 2011) and asserted in cannabis industry claims. In addition, this study explored whether D-limonene modulates other common subjective, cognitive, and physiological effects of THC. Last, this study sought to determine whether D-limonene alone produces any acute drug effects relative to placebo.

2. Methods

2.1. Study Design

The present study utilized a double-blind, within-subjects crossover design. All participants completed a total of nine outpatient drug administration sessions during which they inhaled D-limonene alone (1 mg; 5 mg), THC alone (15 mg; 30 mg), THC and D-limonene together (15 and 30 mg THC + 1 mg D-limonene; 15 and 30 mg THC + 5 mg D-limonene), or placebo (distilled water). After the first eight participants completed the study, an optional 10th test session (30 mg THC + 15 mg D-limonene) was added to the protocol to extend the dose-response curve of D-limonene after appropriate safety data were obtained from the lower doses. The first nine experimental sessions were completed in a randomized order which is standard practice in clinical research to minimize possible order effects and reduce bias (Suresh, 2011). The optional 10th session was administered last because many participants had already begun study participation at the time it was added; participants and research staff were unaware that the 10th session was always the same experimental condition. All sessions were separated by at least 48 hours. This study was conducted at the Johns Hopkins University (JHU) Cannabis Science Laboratory, which is part of the JHU Behavioral Pharmacology Research Unit (BPRU). The protocol was approved by the JHU School of Medicine Institutional Review Board (IRB00085652), approved by the U.S. Food and Drug Administration (IND140339), and was registered on ClinicalTrials.gov (NCT03609853).

2.2. Screening and Experimental Procedures

Participants were recruited for the study using internet advertisements and word-of-mouth communication. Those interested in participating first completed a telephone screening questionnaire. Individuals who appeared eligible based on the initial screening were invited for an in-person evaluation that consisted of a detailed medical history review and physical examination, and routine blood work (i.e., chemistry,

hematology, serology). In addition, participants completed a urine drug test to determine recent drug use an alcohol breathalyzer, and the Timeline Follow-Back to determine drug/alcohol use in the past 90 days (Sobell and Sobell, 1992). Individuals who were deemed eligible based on these screening procedures were randomized into the study (see below for specific inclusion/exclusion criteria). Written informed consent was obtained at the in-person visit prior to any study procedures.

Upon arrival for each session, participants provided a urine sample to test for pregnancy/recent drug use, completed an alcohol breathalyzer, and self-reported their use of drugs/alcohol since their last visit (sessions were not conducted if the participant tested positive for pregnancy, alcohol use, or use of drugs aside from cannabis). Next, participants were fed a standard low-fat breakfast of toast and jam and an intravenous catheter was inserted to facilitate blood sampling throughout the session. After catheter insertion, a baseline blood sample was collected and baseline subjective effects, cognitive performance, and vital signs were assessed.

After baseline assessments, participants inhaled either distilled water (placebo), pure D-limonene, pure THC, or a combination of THC and D-limonene using the Mighty Medic hand-held vaporizer (Storz and Bickel®; see below for drug preparation details). The Mighty Medic was set to a temperature of 210ºC in each study condition. Participants were given 15 minutes to inhale their assigned study drug(s). They were instructed that they should inhale *ad libitum* (i.e., at their own pace), but that they must take a minimum of 15 puffs within the 15 minutes (pilot testing determined that 15 puffs were typically sufficient to exhaust the study doses). Note, the first five participants enrolled in the study (including two individuals who completed all study conditions) used the Foltin paced puffing procedure (Foltin et al., 1987), which was intended to standardize puffing topography. Specifically, this procedure requires participants to take puffs of 5 seconds in duration, followed by a 10 second breath hold, and 40 second inter-puff interval until the dose is exhausted. However, three initial participants who used this procedure experienced adverse effects and were removed from the study prematurely, which led to the adoption of the *ad libitum* procedure described above to allow participants a more comfortable dosing experience and to allow for dose titration in cases in which adverse drug effects began to emerge during drug administration. After each puff, participants exhaled into a handheld smoke filter (Sploofy; City of Industry, CA, USA) to conceal the exhaled aerosol (or vapor) and preserve the study blind, as pre-study testing showed that vapor visibility differed across drug conditions. After the 15th puff, participants exhaled into the open air to determine if the dose had been depleted. If a visible vapor was still observed after the 15th puff, participants were instructed to continue taking puffs until they no longer exhaled a visible vapor (a lack of vapor signified that the dose was depleted). Following drug administration, outcome measures were collected at 15–60 intervals for 6 hours (see Outcome Measures below). Participants were permitted to eat lunch and snacks throughout the experimental session days.

2.3. Participant inclusion/exclusion criteria

Study inclusion criteria were: 1) aged 18–55; 2) good health status as determined by in-person screening (e.g., medical exam, vital signs, routine bloodwork); 3) test negative for drugs of misuse other than cannabis at screening and prior to each study visit; 4) not be pregnant or breastfeeding; 5) have a body mass index (BMI) between 18 and 36 kg/m^2; 6) no allergies to study drugs; 7) self-report previously feeling anxiety after using cannabis on at least one occasion.

Study exclusion criteria included: 1) self-report of non-medical use of psychoactive drugs (aside from cannabis, nicotine, alcohol, or caffeine) in the 3 months prior to study enrollment; 2) history of or current evidence of significant medical condition that could put the participant at risk (e.g., seizure or cardiac disorder); 3) use of prescription or over-the-counter medications (including supplements or vitamins) that could interfere with study results or participant safety; 4) use of dronabinol in the past 30 days; 5) enrollment in another clinical trial in the past 30 days; 6) having sought medical attention (e.g., ER visit) to manage adverse events from cannabis in the past; 7) having anemia or having donated blood in the past 30 days; and 8) use of cannabis, on average, more than twice per week in the past three months.

2.4. Study drug and materials

Synthetic THC (>99% purity) was obtained from THC Pharm GmbH (Frankfurt Am Main, Germany) and was dissolved in pharmacy-grade ethanol (190 proof; Spectrum Chemical, Gardena, CA, USA) to create a solution that was approximately 10% THC/90% ethanol. D-limonene (>99% purity) was obtained from True Terpenes (Hillsboro, OR, USA). Prior to each experimental session, a pharmacist applied a precise amount of D-limonene and/or THC using a micropipette to a steel wool dosing pad, which fit into a small dosing capsule (or "pod") that was placed inside the Mighty Medic vaporizer. The dosing pods containing THC were placed under a fume hood to allow the ethanol to dissipate prior to drug inhalation. Study drugs were added 0.1 mL at a time to ensure the drug solution did not leak through the dosing pad. All study drugs were prepared and dispensed by the Johns Hopkins BPRU Pharmacy.

The THC doses selected (i.e., 15 and 30 mg) were expected to elicit small (15 mg THC) to moderate/large (30 mg THC) anxiogenic responses in healthy adult volunteers based on prior studies (Spindle et al., 2018, 2021). Because there were no published safety data related to the direct inhalation of D-limonene at the outset of the experiment, the initial D-limonene doses (i.e., 1 and 5 mg) were determined based on an analysis of 107 samples of cannabis, representing 29 unique chemovars sold by a licensed cannabis producer in Canada. The mean (range) concentration of D-limonene in the samples tested was 0.11% (0 – 0.45%). Thus, 1 g of cannabis (approximate weight of a single pre-rolled cannabis joint purchased from a dispensary) would, on average, contain about 1 mg of D-limonene, but might contain as high as approximately 5 mg. The optional 10th session (15 mg D-limonene + 30 mg THC) was added after appropriate safety data for direct inhalation of D-limonene were obtained from the first eight study participants.

2.5. Outcome measures

Subjective drug effects, subjective ratings of mood, vital signs, and cognitive performance were assessed at baseline, immediately following drug exposure (i.e., time "0"), and 0.25, 0.5. 0.75, 1, 2, 3, 4, 5, and 6 hours after drug exposure. Study measures were completed in the same order each time. Blood specimens were drawn from an intravenous catheter with 6 mL gray-top vacutainer tubes at baseline, and 0, 0.25, 1, 2, and 3 hours after drug exposure. After collection, specimens were centrifuged (gravitational force, *g*, was 1200 *g*) at 4°C for 10 minutes in order to separate plasma, which was transferred to cryovials for storage at −80ºC until analysis.

2.5.1. Subjective drug effects

A Drug Effect Questionnaire (DEQ) was administered that included 21 items presented individually on a 100-mm visual analog scale ranging from 0 ("not at all") to 100 ("extremely") (Spindle et al., 2021). This questionnaire assessed the overall magnitude of drug effect (e.g., "feel drug effect"), as well as positive (e.g., drug "liking") and negative/adverse effects (e.g., "unpleasant," "sick," "trouble with memory"); of note, three negative/adverse items on the DEQ assessed acute feelings of anxiety (i.e., "paranoid," "anxious/nervous", "heart racing").

2.5.2. Subjective mood state

The 20-item State subscale of the State-Trait Anxiety Inventory (STAI-S) was used to further assess state anxiety/distress before and after drug administration (Spielberger, 1983). Items were presented individually and ranged from 0 (not at all) to 4 (very much so). For each

item, participants rated how they felt "right now, at this moment." A single composite score was generated for this scale, ranging from 20 to 80, with higher scores indicating greater acute anxiety (Spielberger, 1983).

2.5.3. Cognitive performance tasks

Cognitive performance was assessed using two computerized tasks, the Digit Symbol Substitution Task (DSST; McLeod et al., 1982) and the Paced Serial Addition Task (PASAT; Nikravesh et al., 2017). On the DSST, participants replicated the shapes of patterns presented on their screen using a computer keyboard and, on the PASAT, they viewed a string of single-digit numbers and attempted to select the sum of the two numbers most recently presented. The DSST is a test of psychomotor performance and higher order cognition, while the PASAT is a test of working memory (McLeod et al., 1982; Nikravesh et al., 2017). Participants were trained on these tasks during the screening visit to establish a stable baseline and minimize practice effects during experimental sessions. The primary outcome for both tasks was the total number of correct responses.

2.5.4. Vital signs

HR and systolic and diastolic blood pressure (SBP, DBP) were measured in the seated position using an automated monitor.

2.5.5. Pharmacokinetics

Plasma specimens were analyzed for quantitative levels of D-limonene, THC, 11-hydroxy-delta-9-THC (11-OH-THC) and 11-nor-9-carboxy-delta-9-THC (THCCOOH) by iC42 Clinical Research and Development (University of Colorado, Aurora, CO) using gas chromatography-tandem mass spectrometry (GC-MS/MS) and high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) in a College of American Pathologists (CAP) accredited and Clinical Laboratory Improvement Amendments (CLIA) certified laboratory environment. THC and its major metabolites were quantified in EDTA plasma using a previously described, validated LC-MS/MS assay (Klawitter et al., 2017). Limonene EDTA plasma concentrations were measured using a GC-MS/MS assay that was based on a slightly modified, previously validated and published method (Chen et al., 2019). The lower limits of quantitation of the two assays were 0.4 ng/mL for THC, 1.6 ng/mL for 11-OH-THC, 0.4 ng/mL for THCCOOH, and 1 ng/mL for limonene. The upper limit of assay working ranges were 400 ng/mL for THC and its major metabolites and 500 ng/mL for limonene. Results reported here are from analytical runs that met the following predefined acceptance criteria: 75% of the calibrators and 2/3 of the quality controls had an accuracy within 85–115% of the nominal concentrations and imprecision was <15% (coefficient of variance). There was no significant carry over or matrix interferences.

The maximum plasma concentration (C_{max}), area under the plasma concentration curve (AUC), and time to peak plasma concentrations (T_{max}) were determined for each analyte based on a non-compartmental pharmacokinetic analysis using Certara Phoenix Software, version 8.3 (Certara, Princeton, NJ, USA).

2.6. Data presentation and analysis

Demographic characteristics are presented using descriptive statistics, including means and standard deviations. Change-from-baseline data were used to calculate the peak change for subjective effects, cognitive performance, vital signs, and plasma within the first 3 hours post-drug exposure. Pharmacodynamic data were only used for the first 3 hours post-drug exposure because datapoints extending outside of 3 hours likely would have been impacted by nondrug-related factors (e.g., boredom and fatigue).

Peak change-from-baseline scores for each outcome were analyzed using a one-way mixed-effects analysis of variance (ANOVA) with the lone within subject factor of Treatment (Placebo, 1 mg D-Limonene, 5 mg D-Limonene, 15 mg THC, 15 mg THC + 1 mg D-Limonene, 15 mg THC + 5 mg D-Limonene, 30 mg THC, 30 mg THC + 1 mg D-Limonene, 30 mg THC + 5 mg D-Limonene, 30 mg THC + 15 mg D-Limonene). For items in which a significant main effect of treatment was observed, planned comparisons (Fisher's LSD tests) were used to compare: 1) each dose of D-limonene alone (1 and 5 mg) to placebo; 2) THC alone (15 mg) to the corresponding THC/D-limonene combination conditions (i.e., 1 and 5 mg D-limonene + 15 mg THC); 3) THC alone (30 mg) to the corresponding THC/D-limonene combination conditions (i.e., 1, 5, and 15 mg D-limonene + 30 mg THC). For pharmacokinetic outcomes, comparisons were also made between D-limonene alone doses and the corresponding THC/D-limonene combination conditions (e.g., 5 mg D-limonene alone was compared to 5 mg D-limonene + 15 mg THC and 5 mg D-limonene+ 30 mg THC). For all analyses, statistical significance was defined as an alpha level of < 0.05. Analyses were conducted using GraphPad Prism, Version 9 (La Jolla, CA).

3. Results

3.1. Participants

Fifty-three individuals provided informed consent and were screened for the study. Of these individuals, 38 (19 males and 19 females) were eligible and randomized. Of those randomized, 20 (10 males and 10 females) completed the study and were included in data analyses; 12 of these individuals completed the optional 10th experimental session. Of the 18 who are excluded from data analyses, four participants were discontinued due to adverse effects associated with the study drug (one after 30 mg THC, one after 30 mg THC + 1 mg D-limonene, and two after 15 mg THC; three of these participants used the Foltin paced puffing procedure), five were lost to follow-up during study participation, three were discontinued for non-study-related issues (e.g. change in work schedule), one was discontinued due to prohibited non-study drug use (methamphetamine), one was discontinued after starting a new medication, and four were enrolled in the study at the time the laboratory was shut down for seven months due to the COVID-19 pandemic.

The racial/ethnic breakdown of the final study sample (n=20) was: 50% Caucasian/Non-Hispanic, 30% African American/Non-Hispanic, 10% Caucasian/Hispanic, and 10% Asian/Non-Hispanic. Participants did not use nicotine/tobacco, and, on average, had not used cannabis for 116 days (SD = 322; range = 1–1460) prior to their first session. Their mean (SD) BMI was 26 kg/m^2 (6), their mean weight was 168 lbs (39), and their mean age was 26 years old (4; range: 18–40 years old).

3.2. Adverse events

In total, there were 14 adverse events (AEs) spontaneously reported by participants in the study, none of which were considered unanticipated or serious. Of these 14 cases, participants primarily experienced dizziness/lightheadedness (8 instances; two in the 30 mg THC + 1 mg D-limonene condition, two in the 30 mg THC + 5 mg D-limonene condition, one in the 15 mg THC + 1 mg D-limonene condition, one in the 30 mg THC condition, one in the 1 mg D-limonene condition, and one in the 5 mg D-limonene condition) and/or anxiety-like effects (6 instances; two in the 30 mg THC + 1 mg D-limonene condition, two in the 15 mg THC condition, one in the 30 mg THC condition, and one in the 1 mg D-limonene condition); note that the three adverse events that occurred in the D-limonene alone conditions happened prior to drug administration and were related to the IV catheter insertion and baseline blood draw. One participant experienced sedation, nausea, and emesis following drug administration (30 mg THC condition).

3.3. Subjective drug effects

Fig. 1**A-C** illustrates the mean (± SEM) subjective ratings of "anxious/nervous" over time and peak change-from-baseline scores for

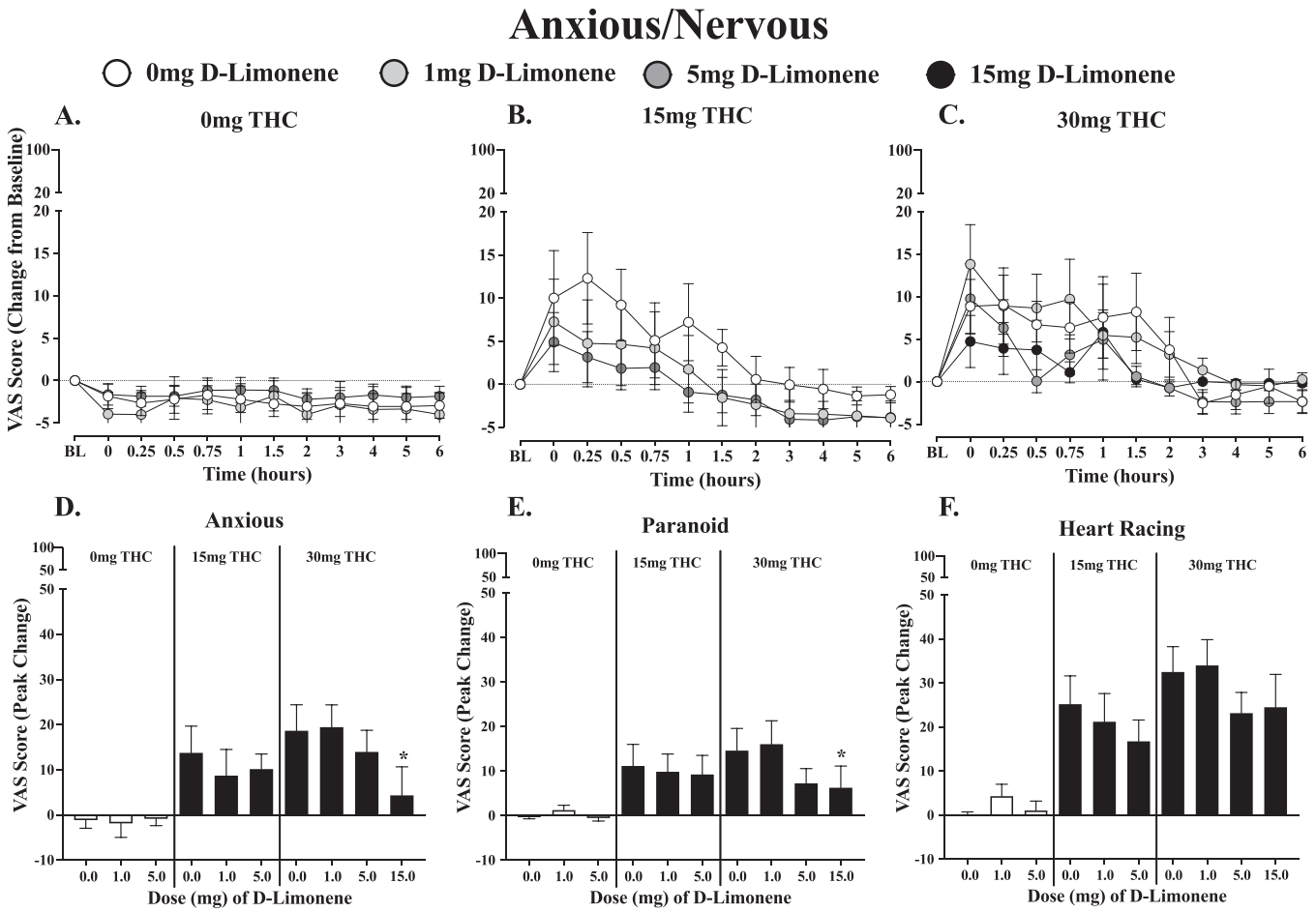

Fig. 1. Time course for mean (SEM) subjective ratings of "anxious/nervous are shown for conditions with 0 mg THC (**A**), 15 mg THC (**B**), and 30 mg THC (C). Mean (SEM) peak change from baseline ratings for the visual analog scale (VAS) items **D)** anxious/nervous, **E)** paranoid, and **F)** heart racing from the Drug Effect Questionnaire (DEQ). VAS scores ranged from 0 (not at all) to 100 (extremely). Filled bars indicate conditions with active THC administration. * indicates a significant difference from the THC alone condition within a given THC dose (i.e., 15 or 30 mg THC). Note that the 30 mg THC/15 mg limonene condition only included 12 participants (20 participants completed all other study conditions).

the three DEQ items considered to be subjective indices of anxiety or panic-like experiences ("anxious/nervous," "paranoid," and "heart racing"; Fig. 1**D-E**). A main effect of Treatment was observed for "anxious/nervous" (F[4.197, 76.02]=4.026; $p<0.01$), "paranoid" (F[4.230, 76.61]=3.601; $p<0.01$), and "heart racing" (F[4.965, 89.92]=6.994; $p<0.0001$). Planned comparisons revealed that ratings of "anxious/nervous" and "paranoid" were significantly lower in the 30 mg THC + 15 mg D-limonene condition compared with the 30 mg THC alone condition (p's < 0.05); none of the other planned comparisons were significantly different.

On the STAI-S questionnaire (composite score), there was a significant main effect of Treatment (F[4.798, 86.89]=3.476; $p<0.01$; Fig. 2). Although planned comparisons did not meet the *a-priori* threshold for statistical significance, reductions in anxiety were dose-orderly and approached significance for the 30 mg THC + 15 mg D-limonene condition compared with 30 mg THC alone ($p=0.08$).

Fig. 3 illustrates the mean (± SEM) peak change-from-baseline scores for subjective ratings of "feel drug effect" (Fig. 3**A**), "pleasant drug effect" (Fig. 3**B**) and "unpleasant drug effect" (Fig. 3**C**). There was a significant main effect of Treatment for all three items (p's < 0.05). Planned comparisons revealed that 30 mg THC + 15 mg D-limonene produced significantly lower subjective ratings of "unpleasant" compared with 30 mg THC alone ($p=0.03$); none of the other planned comparisons were significantly different for these items.

Other items on the DEQ that are commonly impacted by acute THC

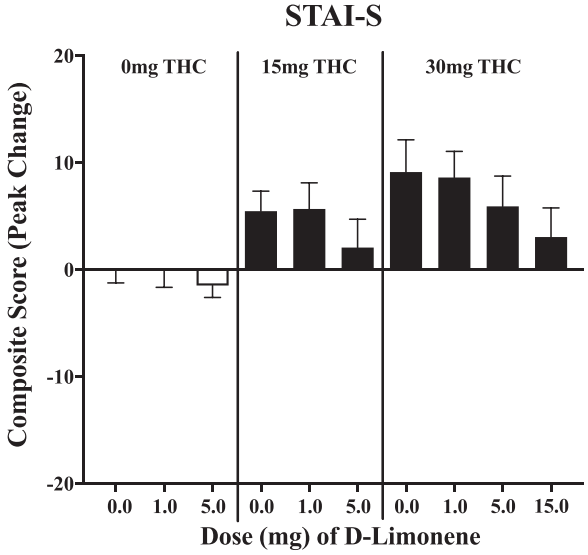

Fig. 2. Mean (SEM) peak change from baseline composite scores on the State-Trait Anxiety Inventory – State questionnaire (STAI-S). Greater scores indicate higher anxiety. Filled bars indicate conditions with active THC administration. Note that the 30 mg THC/15 mg limonene condition only included 12 participants (20 participants completed all other study conditions).

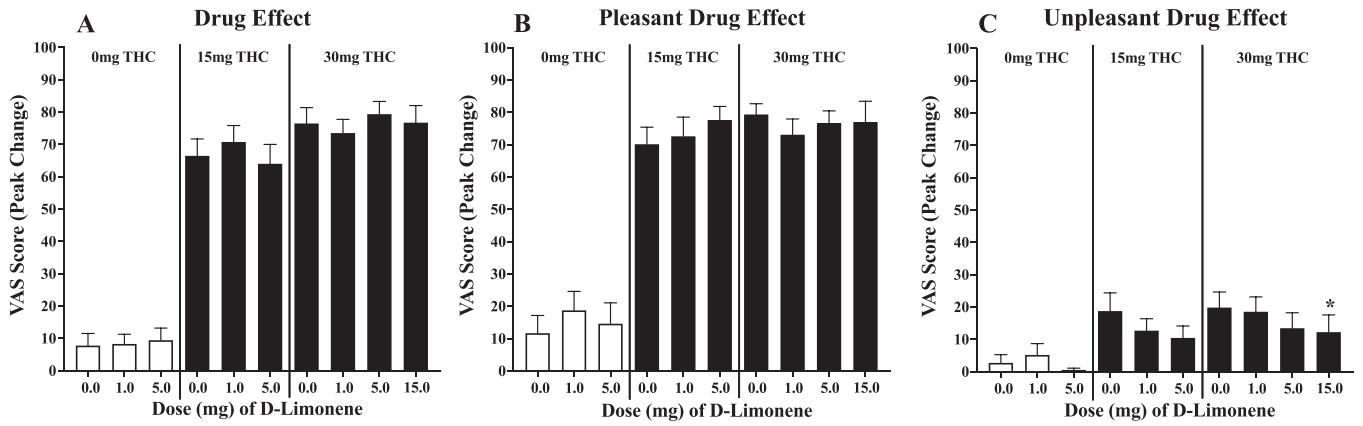

Fig. 3. Mean (SEM) peak change from baseline ratings for the visual analog scale (VAS) items **A)** drug effect, **B)** pleasant drug effect, and **C)** unpleasant drug effect from the Drug Effect Questionnaire (DEQ). Scores ranged from 0 (not at all) to 100 (extremely). Filled bars indicate conditions with active THC administration. * indicates a significant difference from the THC alone condition within a given THC dose (i.e., 15 or 30 mg THC). Note that the 30 mg THC/15 mg limonene condition only included 12 participants (20 participants completed all other study conditions).

exposure were increased, as expected, when THC or THC + D-limonene was administered relative to placebo, but ratings did not differ between THC and THC + D-limonene conditions (see Table 1). Additionally, 1 mg and 5 mg of D-limonene alone did not produce any significant changes on subjective ratings compared with placebo.

3.4. Cognitive Effects

Fig. 4 illustrates the mean peak change-from-baseline total correct for the DSST (Fig. 4**A**) and PASAT (Fig. 4**B**) for each experimental condition. Following drug administration, a significant main effect of Treatment was observed for the DSST (F[6.276, 113.7]=2.560; $p<0.05$) but not for the PASAT (F[4.856, 87.95]=1.465; p=0.21). Planned comparisons did not detect any significant differences between THC and THC/D-limonene combination conditions or between D-limonene alone and placebo.

3.5. Vital Signs

Fig. 4C illustrates the peak change-from-baseline data (i.e., beats per

Table 1
Peak changes in pharmacodynamic outcomes using change-from-baseline data.

Outcome Measure	0 mg THC +			15 mg THC +			30 mg THC +			
	0 mg D-limonene	1 mg D-limonene	5 mg D-limonene	0 mg D-limonene	1 mg D-limonene	5 mg D-limonene	0 mg D-limonene	1 mg D-limonene	5 mg D-limonene	15 mg D-limonene
Subjective Measures										
DEQ										
Drug Effect	8 (17)	8 (14)	10 (17)	67 (23)	71 (23)	64 (27)	77 (22)	74 (19)	79 (18)	77 (18)
Unpleasant	3 (11)	5 (16)	1 (3)	19 (25)	13 (17)	11 (17)	20 (22)	19 (21)	14 (21)	12 (19)*
Pleasant	12 (25)	19 (26)	15 (29)	70 (24)	73 (27)	78 (19)	79 (15)	73 (22)	77 (17)	77 (22)
Drug Liking	17 (31)	18 (27)	19 (32)	63 (33)	68 (26)	69 (36)	73 (23)	72 (23)	72 (26)	72 (28)
Sick	0 (1)	3 (12)	-1 (4)	6 (12)	4 (8)	5 (12)	4 (11)	12 (22)	9 (17)	12 (23)
Heart Racing	0 (3)	4 (12)	1 (10)	25 (29)	21 (29)	17 (22)	33 (26)	34 (26)	23 (21)	25 (26)
Anxious/Nervous	-1 (8)	-2 (14)	-1 (7)	14 (27)	9 (26)	10 (15)	19 (26)	20 (22)	14 (22)	4 (22)*
Relaxed	-10 (40)	-7 (49)	-9 (42)	-1 (51)	-9 (45)	-8 (47)	2.1 (54)	-12 (42)	-10 (52)	20 (53)
Paranoid	0 (2)	1 (5)	-1 (3)	11 (22)	10 (18)	9 (19)	15 (22)	16 (23)	7 (15)	6 (17)*
Sleepy/Tired	8 (43)	4 (40)	9 (39)	47 (35)	36 (45)	49 (35)	35 (47)	20 (57)	33 (50)	49 (39)
Alert	-32 (37)	-6 (36)	-29 (39)	-32 (43)	-13 (46)	-43 (34)	-16 (58)	-36 (33)	-40 (29)	-26 (39)
Irritable	0 (1)	3 (14)	0 (0)	7 (15)	10 (15)	4 (10)	7 (13)	9 (15)	5 (9)	1 (5)
Vigorous/Motivated	-19 (46)	-9 (37)	-31 (39)	-16 (39)	-11 (37)	-18 (42)	-4 (44)	-15 (43)	-22 (41)	-17 (49)
Restless	0 (14)	4 (12)	-5 (16)	18 (22)	11 (20)	5 (28)	26 (28)	24 (27)	11 (27)*	19 (28)
Hungry/Have Munchies	14 (38)	28 (22)	13 (38)	36 (44)	42 (44)	45 (39)	34 (45)	53 (30)*	46 (46)	52 (32)
Cannabis Craving	-7 (15)	3 (10)	-2 (17)	6 (24)	4 (10)	6 (20)	3 (32)	-1 (26)	6 (31)	-4 (24)
Dry Mouth	3 (12)	6 (12)	-1 (15)	37 (29)	37 (31)	37 (27)	44 (27)	37 (38)	47 (27)	21 (35)
Dry/Red Eyes	3 (7)	6 (13)	-1 (7)	25 (28)	26 (30)	25 (30)	37 (30)	25 (25)	35 (24)	32 (31)
Memory Impairment	1 (3))	3 (9)	1 (5)	12 (18)	14 (18)	21 (5)	21 (20)	24 (25)	15 (20)	21 (25)
Throat Irritation/Coughing	1 (2)	1 (5)	1 (6)	18 (23)	17 (26)	18 (22)	21 (22)	24 (28)	35 (35)*	26 (33)
Difficulty Performing Routine Tasks	3 (7)	4 (11)	1 (2)	23 (32)	26 (29)	24 (27)	36 (30)	25 (29)	29 (31)	24 (31)
Cognitive Performance Measures										
DSST: Total Correct	-1 (10)	2 (11)	-4 (13)	-7 (14)	-7 (16)	-9 (18)	-9 (14)	-12 (13)	-6 (17)	-10 (13)
PASAT: Total Correct	-7 (20)	-2 (13)	-11 (25)	-16 (22)	-14 (19)	-11 (22)	-15 (26)	-16 (26)	-14 (22)	-15 (12)
Physiological Measures										
Heart Rate, beats/min	1 (18)	0 (13)	-1 (14)	17 (21)	20 (25)	20 (20)	25 (23)	24 (25)	29 (10)	28 (14)
Diastolic Blood Pressure, mmHg	-6 (14)	-5 (16)	-3 (17)	9 (20)	-2 (20)	-3 (17)	4 (18)	3 (22)	-2 (18)	-3 (22)
Systolic Blood Pressure, mmHg	-4 (18)	-6 (19)	13 (19)	4 (20)	-2 (22)	-10 (21)	0 (23)	-6 (21)	-7 (17)	6 (27)

Abbreviations: DEQ = Drug Effect Questionnaire, DSST = Digit Symbol Substitution Task, PASAT = Paced Serial Addition Task. SD = Standard Deviation
* indicates a significant difference from the within THC dose alone condition (p's<0.05)

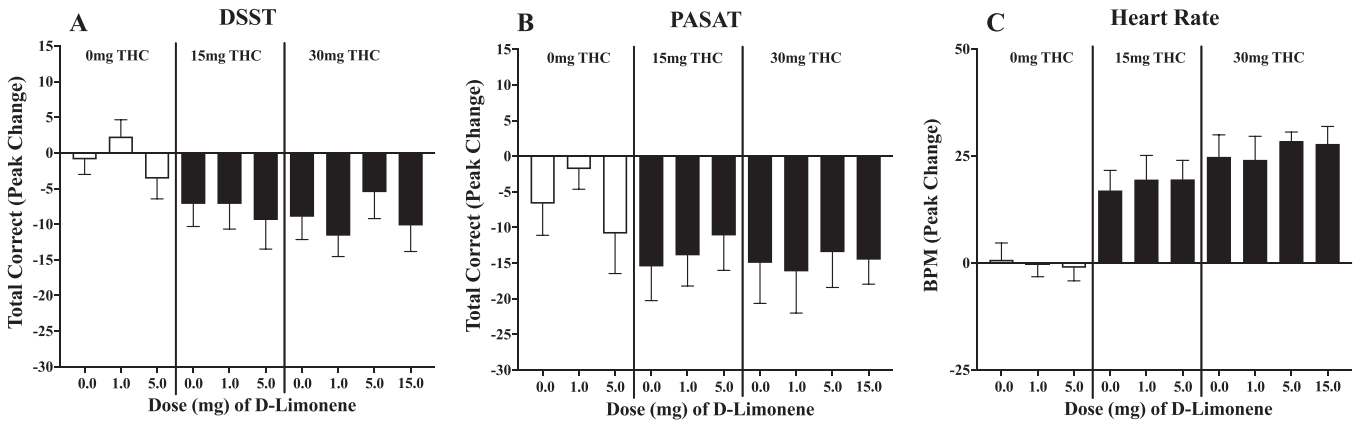

Fig. 4. Mean (SEM) peak change from baseline ratings for the total correct on the **A**) DSST and **B**) PASAT, the two cognitive performance measures in the study. A decrease in total correct indicates an impairment of cognitive/psychomotor function. Filled bars indicate conditions with active THC administration. Note that the 30 mg THC/15 mg limonene condition only included 12 participants (20 participants completed all other study conditions).

minute) for HR. A main effect of Treatment was detected for HR (F[4.370, 79.15]=9.170; p<0.0001). Planned comparisons revealed that there were no differences within each THC dose condition (e.g., no difference between 30 mg THC vs 30 mg THC + 15 mg D-limonene) nor between d-limonene alone versus placebo conditions. There were no main effects of Treatment detected for SBP or DBP (p's>0.05).

3.6. Plasma THC and D-limonene concentrations

Fig. 5 depicts the mean peak change-from-baseline plasma concentrations (i.e., C_{max}) for THC and D-limonene for each experimental condition. Full time course data for D-limonene, THC, and 11-OH-THC are shown in **Supplemental** Fig. 1. A main effect of Treatment was detected for THC (F[1.113, 20.15]=15.56; p<0.01). Planned comparisons revealed that 30 mg THC + 15 mg D-limonene produced significantly greater concentrations of THC relative to 30 mg THC alone (p<0.05); no other differences were observed between THC alone versus THC/D-limonene combination conditions (Fig. 5**A**). There was a significant main effect of Treatment for concentrations of D-limonene (F[3.896, 71.12]=51.71; p<0.0001). Planned comparisons revealed that there was a dose-dependent increase in D-limonene within a given THC group (p's<0.05). Further, planned comparisons revealed that 5 mg D-limonene combined with 15 mg and 30 mg THC produced significantly greater concentrations of D-limonene relative to 5 mg D-limonene alone (p's<0.05).

4. Discussion

To date, little controlled clinical research has been conducted to evaluate hypothesized interactions between THC and various terpenes that are often included in marketing and product labeling by the cannabis industry. This experiment showed that simultaneously administering vaporized D-limonene and THC reduced subjective indices of THC-induced anxiety in a dose-orderly manner. However, co-administration of D-limonene with THC did not systematically alter other subjective, cognitive, or physiological effects of THC, and D-limonene alone did not elicit any pharmacodynamic effects when compared with placebo. Overall, these results are consistent with prior preclinical research described in the introduction which similarly demonstrated an anxiolytic effect of D-limonene (Carvalho-Freitas and Costa, 2002; Komiya et al., 2006; Song et al., 2021) and corroborate historical references to ingestion of lemon or citrus juices as antidotes to overdoses of cannabis or hashish (as documented in Russo, 2011).

The present study provides the first empirical clinical evidence that

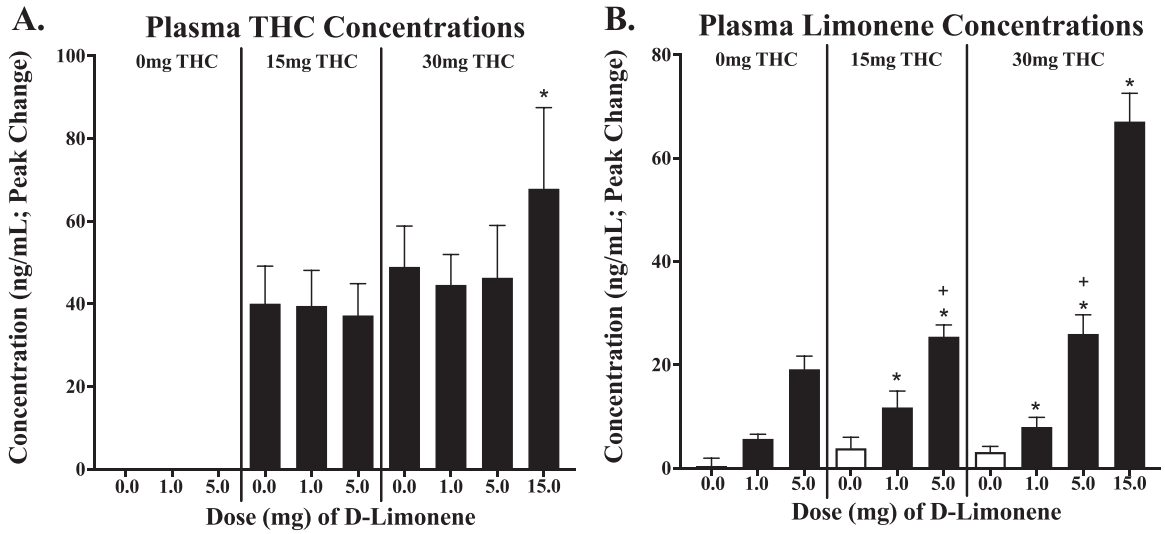

Fig. 5. Mean (SEM) maximum plasma concentrations, or Cmax, in ng/mL for **A**) THC and **B**) D-Limonene. * indicates a significant difference from the THC alone condition within a given THC dose (i.e., 15 or 30 mg THC). + indicates a significant difference from the D-limonene alone condition within a given D-limonene dose (i.e., 1 or 5 mg D-limonene). Note that the 30 mg THC/15 mg limonene condition only included 12 participants (20 participants completed all other study conditions).

D-limonene can attenuate acute anxiogenic effects of relatively high doses of inhaled THC. The observed reduction in THC-induced anxiety is noteworthy given that acute anxiety/paranoia is one of the most common adverse effects associated with the use of cannabis products and synthetic THC pharmaceuticals (e.g., Dronabinol, Nabilone; Bajtel et al., 2022; Drennan et al., 2021; Lewandowska et al., 2021; Sharpe et al., 2020; Zamarripa et al., 2022). Indeed, acute anxiety/paranoia is one of the chief symptoms among patients who present for emergency medical evaluation related to cannabis-induced intoxication (Keung et al., 2023; O'Brien et al., 2022; Randall et al., 2020) and these side effects have also been cited as a reason that some individuals discontinue the use of medicinal cannabis (Martin et al., 2021). The results of the present study suggest that the development of novel cannabis product formulations high in D-limonene could be a viable and relatively straightforward strategy to widen the therapeutic window of medicinal cannabis and/or THC and potentially reduce adverse effects associated with non-medicinal cannabis use.

The pharmacological mechanism by which D-limonene exerts its anxiolytic effects and interacts with THC remains somewhat unclear given the limited research that has been completed to date. Prior preclinical studies using rodent models of stress have posited that D-limonene exerts its anxiolytic effects via a combination of GABAergic, dopaminergic, and serotonergic mechanisms (Komiya et al., 2006), and one study suggested anxiolytic effects of D-limonene were driven by adenosine (A_{2A}) receptor-mediated increases in dopamine and GABA concentrations in the striatum (Song et al., 2021). Preclinical receptor binding studies indicate that D-limonene does not directly alter the function of THC at CB_1 or CB_2 receptors (Santiago et al., 2019) or transient receptor potential ankyrin 1 (TRPA1) or transient receptor potential vanilloid (TRPV1) channels (Heblinski et al., 2020). Thus, the observed interaction between D-limonene and THC on acute anxiety was most likely driven by unique effects of D-limonene as opposed to modulation of THC's pharmacology by D-limonene, though more research in this area is warranted.

Importantly, the anxiolytic effects of D-limonene in this study were most evident at 15 mg and at a 2:1 ratio of THC to D-limonene. Our prestudy evaluation of the chemical composition of retail cannabis samples (see Methods) suggests this amount of D-limonene and THC:D-limonene ratio is unlikely to be encountered in unadulterated cannabis flower products. Thus, the extent to which the results from the present study may generalize to retail cannabis products is unclear. Another important consideration is that pure THC and D-limonene were used in this study as opposed to whole-plant THC-dominant cannabis with varying concentrations of D-limonene, as is more likely to be encountered in the retail market. Because there are hundreds of other chemical constituents in the cannabis plant that can potentially interact through multiple pharmacological mechanisms, it is unclear whether simply increasing the concentration of D-limonene in cannabis cultivars or reconstituting whole-plant or "full-spectrum" cannabis products with added D-limonene would achieve the same results as those observed here. Additional studies are needed to replicate and extend the findings from the present study to elucidate the THC:D-limonene ratios and doses that optimize anxiety reduction and to evaluate whether the effects observed here extend to other routes of administration (e.g., smoked or oral dosing) and product types (e.g., whole plant or "full spectrum" cannabis products). Replication of the present research with oral dosing is especially important given that people who use cannabis medicinally typically prefer oral ingestion over inhalation (Boehnke et al., 2019; Spindle et al., 2019) and given that the only FDA-approved pharmaceutical preparations of THC are oral dose formulations.

In addition to the study limitations mentioned above (i.e., use of isolated compounds and one route of administration), another important limitation to the present study was that the 30 mg THC + 15 mg D-limonene condition was always completed last and was only completed by 12 of 20 participants; this design limitation was necessitated by a lack of safety data for the direct inhalation of vaporized D-limonene at the outset of the study. Because this dose condition was not randomized, it is possible order effects or the development of tolerance to THC may have impacted our findings. That said, we believe our conclusions are valid due to the following observations. First, overall, the effect of D-limonene on THC-induced anxiety responses were dose orderly and similarly observed (albeit to a lesser extent) in the 30 mg THC + 5 mg D-limonene dose condition, which was randomized. Second, there were no differences observed on other pharmacodynamic outcomes (e.g., non-anxiogenic subjective effects, cognitive performance), suggesting THC tolerance did not develop over the course of the experiment. Taken together, these observations suggest that the data from the present study demonstrate a real effect of D-limonene in attenuating THC-induced anxiety as opposed to an artifact of the study design, but replication of these findings is encouraged. An additional limitation of note is that we were underpowered to explore possible sex differences across study conditions.

In summary, the present controlled human laboratory study found that the cannabis terpene D-limonene attenuated THC-induced anxiety in a dose-orderly fashion, but had little impact on other common acute subjective, cognitive, or physiological effects of THC in this sample of healthy adults. Moreover, when inhaled alone, D-limonene did not produce any acute effects that differed from placebo. This is among the first clinical studies to demonstrate the validity of the cannabis entourage effect, which theorizes that THC and other constituents of the plant interact in meaningful ways that alter acute cannabis effects. Given the growing interest in the use of cannabis for medicinal purposes and expanding legalization of cannabis for nonmedicinal purposes, further understanding of which constituents may increase the safety profile of cannabis by attenuating acute adverse effects (e.g., anxiety and paranoia), and which constituents may exacerbate adverse effects, is paramount for advancing the use of cannabinoids in medicine and, more broadly, protecting public health.

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Author contributions

Tory Spindle and Austin Zamarripa conducted statistical analyses, created graphs/tables, and completed the first draft of the manuscript. Alexandra Ward, Bridget Tomspon, Cristina Sempio, Touraj Shokati, Jost Klawitter, and Uwe Christians contributed to the pharmacokinetic methods section and performed pharmacokinetic analyses. Remaining authors contributed to the final draft of the manuscript by providing comments and feedback and all authors approve of the contents of the final manuscript.

CRediT authorship contribution statement

Ethan Russo: Writing – review & editing, Conceptualization. **C. Austin Zamarripa:** Writing – original draft, Formal analysis. **Tory Spindle:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Bridget Tompson:** Writing – review & editing. **Alexandra Ward:** Writing – review & editing, Project administration. **George Bigelow:** Writing – review & editing, Funding acquisition, Conceptualization. **Lauren Pollak:** Writing – review & editing, Project administration. **Uwe Christians:** Writing – review & editing, Writing – original draft. **Jost Klawitter:** Writing – original draft. **Touraj Shokati:** Writing – review & editing, Project administration. **Cristina Sempio:** Writing – review & editing. **Ryan Vandrey:** Writing – review & editing, Writing – original draft, Funding acquisition,

Conceptualization.

Declaration of Competing Interest

Dr. Tory Spindle has served as a consultant for Canopy Health Innovations Inc. and has received research funding from Cultivate Biologics. Dr. Ryan Vandrey has served as a consultant or received honoraria from Mira1a Therapeutics Inc., Jazz Pharmaceuticals, Charlotte's Web, Syqe Medical Ltd., and WebMD. Dr. Ethan Russo is the founder and CEO of CReDo Science and a scientific advisor to True Terpenes. A patent application (PCT/US2022/014296) has been submitted by Johns Hopkins University on behalf of Drs. Vandrey, Spindle, and Russo for the use of d-limonene to reduce THC-induced anxiety based on the data presented in this manuscript (the submission occurred after the trial had concluded and data was analyzed). Minimization of bias was ensured by posting of the trial on ClinicalTrials.gov before it commenced. The study was conceived and designed by Drs Vandrey and Russo. Drs. Spindle, Vandrey, and Zamarripa had full access to the data and worked together to write the first draft of the manuscript and each take responsibility for the integrity of the reported data. Remaining authors contributed to the manuscript by reviewing the completed draft and providing critical feedback and edits. Johns Hopkins played no role in the production of this manuscript. The remaining authors have no conflicts of interest to declare.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.drugalcdep.2024.111267.

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