Heliyon 5 (2019) e01915

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.heliyon.com

The sesquiterpene beta-caryophyllene oxide attenuates ethanol drinking and place conditioning in mice

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ARTICLE INFO

Keywords:
Biochemistry
Anatomy
Immunology
Neuroscience
Physiology
Toxicology

ABSTRACT

Approximately 20 million adults in the United States have an alcohol use disorder. In recent years, modulation of the behavioral effects of ethanol by phytochemicals has been explored. In this study, we used the ethanol-induced loss of righting reflex (LORR) assay to assess potency differences between the sesquiterpene phytochemical beta-caryophyllene (BCP) and its derivative caryophyllene oxide (BCPO). We also investigated the effects of BCPO on two bottle-choice ethanol drinking and the ethanol-induced conditioned place preference (CPP). We then determined whether there are any pharmacokinetic or pharmacodynamic interactions between BCPO and ethanol, using blood ethanol analysis and pretreatments with the selective cannabinoid receptor 2 (CB2) antagonist AM630, respectively. BCPO augmented the ethanol-induced LORR at a dose (30 mg/kg) tenfold lower than BCP (300 mg/kg). Swiss-Webster mice were found to split into stable high and low drinking groups. This same dose (30 mg/kg) of BCPO significantly decreased ethanol intake and preference for ethanol over water in mice that consumed high amounts of ethanol, without any effect on total fluid intake. BCPO had limited effects in mice that consumed low amounts of ethanol. BCPO also significantly attenuated the ethanol-induced CPP. Blood ethanol analysis showed no significant effect of ethanol on the pharmacokinetics of ethanol. Furthermore, the enhancement of the ethanol-induced LORR by BCPO was reversed by AM630. These findings demonstrate that BCPO more potently modulates the behavioral effects of ethanol than the parent compound BCP. Moreover, they suggest that BCPO modulates the behavioral effects of ethanol through pharmacodynamic rather than pharmacokinetic mechanisms.

1. Introduction

Alcohol use disorder (AUD) is a major public health concern affecting a significant proportion of the US population. AUD has a yearly prevalence of 13.9% and a lifetime prevalence of 29.1% (Grant et al., 2015). Current treatments for AUD yield disappointing remission rates, spurring the need for new targets and treatments. Plants and plant extracts may provide compounds that can be used to validate novel targets for the treatment of AUD, as they have provided compounds useful in the development of treatments for other brain disorders such as Alzheimer's disease (Howes and Houghton, 2003). In support of this, studies have examined plant derivatives and phytochemicals such as *Hypericum perforatum* (St. John's Wart), isoflavonoids from *Pueraria lobata* (kudzu), ibogaine, the dietary cannabinoid β-caryophyllene (BCP) in preclinical animal studies with a focus on AUD. These studies have investigated various aspects of alcohol intake or alcohol-seeking behavior including voluntary alcohol consumption, ethanol preference and ethanol-induced place conditioning (Al Mansouri et al., 2014; Rezvani et al., 2003) Hence, there is a sound basis for the study of phytochemicals and plant extracts in the development of promising targets for the treatment of AUD.

Plant extracts have been shown to reduce ethanol intake by themselves as well as synergistically with pharmaceuticals. An extract of *Hypericum perforatum* has been shown to reduce ethanol intake in alcohol preferring rats without affecting food, water, or saccharin intake, in a dose range of 30–130 mg/kg. A subthreshold dose (1 mg/kg) of the opioid receptor antagonist naloxone augmented the suppression of ethanol intake by this extract (Perfumi et al., 2003). Likewise, the phytochemical BCP has been shown to reduce voluntary alcohol intake

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https://doi.org/10.1016/j.heliyon.2019.e01915
Received 8 February 2019; Received in revised form 5 May 2019; Accepted 3 June 2019

and attenuate the ethanol-induced conditioned place preference (CPP) in mice (Al Mansouri et al., 2014). It is important to note, however, that although BCP is known to activate cannabinoid type 2 (CB2) receptors (Gertsch et al., 2008), the precise mechanism through which it interacts with ethanol remains speculative.

BCP and its derivative β-caryophyllene oxide (BCPO) are naturally occurring sesquiterpenes found in reasonable quantities in the essential oils of many plants including clove (*Syzygium aromaticum),* rosemary *(Rosmarinus officinalis),* black pepper *(Piper nigrum),* and marijuana *(Cannabis sativa),* among others (Ghelardini et al., 2001; Ormeno et al., 2008). BCP and BCPO have been approved by the US Food and Drug Administration and the European Food and Safety Authority as food additives. Both BCP and BCPO are generally regarded as tolerable, safe, and non-toxic. BCP has been shown to possess antioxidant, anti-inflammatory and analgesic properties (Klauke et al., 2014), while BCPO has been shown to exhibit many useful characteristics, including antifungal, analgesic, and anti-inflammatory properties (Chavan et al., 2010; Yang et al., 1999). However, no previous study has examined whether BCPO, like its parent compound BCP, decreases ethanol drinking or place conditioning. Moreover, previous *in vitro* research suggests that it did not have appreciable affinity at CB2 receptors (Gertsch et al., 2008).

To elucidate these areas, in the present study, we compared the potency of BCP and BCPO using the loss of righting assay. We examined whether BCPO reduces ethanol drinking and attenuates ethanol-induced place conditioning. We then determined whether there are any pharmacokinetic or pharmacodynamic interactions between BCPO and ethanol, using blood ethanol analysis and pretreatments with the selective CB2 receptor antagonist AM630, respectively. We hypothesized that BCPO, like the parent compound BCP, would attenuate ethanol drinking and place conditioning.

2. Materials and methods

2.1. Drugs

BCPO was extracted at the Research Triangle Institute. BCPO and was suspended in 10% tween and saline using sonication. BCP was purchased from Sigma-Aldrich (St. Louis, MO) at 89% purity. BCP was diluted in olive oil. AM630 was synthesized at Northeastern University. AM630 was dissolved in 2.5% DMSO and saline. OmniPur®, pure 200 proof, ethanol was commercially purchased (VWR, Radnor, PA) and was diluted with saline for intraperitoneal injection. For oral consumption, OmniPur®, pure 200 proof was diluted with tap water to the desired final concentration.

2.2. Animals

Male Swiss-Webster mice (CFW; Charles River Laboratories, Inc.; Wilmington, MA) weighing between 30–50 g served as the subjects of these experiments. Swiss-Webster mice were chosen for these studies because these mice are a general purpose strain that has been used extensively to study behavior, physiology, and neurochemistry (Goeders et al., 2009; Murnane, 2018; Murnane et al., 2009, 2012, 2019; Murphy and Murnane, 2019; Oppong-Damoah et al., 2019; Ray et al., 2018). In the context of ethanol research, we have consistently found that mice from this strain acquire two distinct and stable phenotypes of ethanol consumption, with approximately 50% of mice drinking more than 10 g/kg of ethanol per day and 50% drinking less than 10 g/kg of ethanol per day. This allows for the study of drug treatments and brain mechanisms in two discrete phenotypes (i.e., high and low ethanol consumers). The mice were housed in groups of 4 for all experiments, except for the two-bottle choice experiments, in which they were housed singly. All mice were maintained in a temperature and humidity-controlled room on a 12-hour light/dark cycle. The lights were turned off at 7pm every evening and turned back on at 7am every morning. Animals had access to food (Laboratory Rodent Diet) and water *ad libitum*. The bedding in the housing boxes was changed every Friday for all experimental animals with the exception of mice in the two-bottle choice experiments, whose bedding was changed every Saturday after consumption readings were taken. All animals employed in this study were treated according to protocols evaluated and approved by Institutional Animal Care and Use Committee of Mercer University.

2.3. Loss of righting reflex (LORR)

The LORR assay is a behavioral model that measures a direct elicited effect of ethanol (i.e., sedation). We chose to implement this assay using a repeated-measures design to reduce the number of mice necessary to complete the experiments and reduce the influence of between-subject variability. The LORR procedure was conducted using previously described procedures (Al Mansouri et al., 2014). Briefly, mice were given an injection of vehicle, BCP, or BCPO 15 minutes prior to the injection of ethanol at 3.0 g/kg, which was established as a sedative dose in our unpublished preliminary studies. AM630 was administered 15 minutes prior to BCPO injections. Each mouse was placed in the supine position in a plastic trough 10 minutes after the ethanol injection. The time to recover from the ethanol-induced LORR was continuously recorded. A single displacement from the supine position was not used as the criteria for recovery LORR as this may not reflect a complete recovery of the reflex. If a mouse righted itself at any point in the experiment, it was placed again in the supine to observe an additional righting reflex. The criteria for LORR recovery was the ability of the mouse to right themselves three times within 30 seconds. For the test drugs, an ascending order was used for the repeated-measures dosing, with one set of animals in the BCP study and a separate set of age, sex, housing density, weight, and strain matched animals in the BCPO study. Mice were injected once per week with BCP (30, 100, 178 and 300 mg/kg) or BCPO (1, 10, and 30 mg/kg) starting with the lowest dose and working up to the first dose that significantly increased the ethanol-induced LORR.

2.4. Two-bottle choice ethanol preference test

The two-bottle choice paradigm is an animal model that captures aspects of voluntary alcohol consumption. Intermittent access to 20% ethanol has been described to induce high rates of ethanol consumption (Hwa et al., 2011), and was therefore utilized for this study. The experiment began by isolating the mice to record drinking data from each subject. The following day, the large single bottle of water available in the home box was replaced with two 25ml pipettes fitted with stainless steel metal sippers with each one containing water. Each mouse was allowed to acclimate to drinking from two bottles of water for 10 days. The intermittent provision of ethanol in one of the two 25 ml pipette bottles was then initiated, with ethanol available on Monday, Wednesday and Friday. The procedures that we followed have been previously described (Hwa et al., 2011). Briefly, a 3%, 6% and 10% (w/v) of ethanol solution was presented on the first Monday, Wednesday and Friday of the acquisition of ethanol drinking. The following Monday, and for the remainder of the study, mice received 20% ethanol in one pipette and water in the other pipette. The bottles were changed every day, and were changed between one and three hours before the beginning of the dark cycle to further enhance drinking rates (Thiele and Navarro, 2014). The placement of the pipettes was alternated in each drinking session to prevent the development of a side preference. Each animal was weighed before each drinking session to accurately calculate the ethanol consumption in g/kg. A “spill cage” (cage with no animal) was set up as described previously (Hwa et al., 2011) to control for spillage as a result of experimenter handling of the boxes and pipettes or because of evaporation. Acquisition was complete when a stable drinking pattern emerged, which was operationally defined as consumption (in g/kg) that varied by less than 20% across at least 4 consecutive sessions. This typically occurred after 9 sessions of 20%v/v ethanol (i.e., three weeks of

intermittent access). We have consistently found that Swiss-Webster mice naturally develop stable drinking patterns with approximately 50% of mice drinking more than 10 g/kg of ethanol per day and 50% drinking less than 10 g/kg, which we describe as high and low drinkers, respectively. After a stable drinking pattern was observed, on Mondays, mice (N = 18) were injected with BCPO (30 mg/kg) or vehicle 30 minutes prior to ethanol introduction. This was alternated the following week. Ethanol consumption and preference as well as total fluid consumption was assessed 1 hour and 24 hours following provision of ethanol.

2.5. CPP

CPP is used to assess the positive appetitive conditioned effects of a drug (Fantegrossi et al., 2008). In this study, CPP experiments were performed using a three compartment CPP chamber (Med Associates, St. Albans, VT) placed individually in soundproof boxes (Med Associates, St. Albans, VT). We used an expedited CPP process that has been described previously (Calcagnetti and Schechter, 1992). Mice (N = 8 per treatment or dose group) were habituated for 2 days in the CPP chamber for 30 minutes each day where they had unrestricted access to all sides of the chamber. On the third day, mice were placed in the chamber for a 15-minute pretest with unrestricted access to establish which environment was preferred. Mice were then conditioned twice a day for four consecutive days. One side of CPP chamber contained black walls and a smoother floor and the other side contained white walls with a metal mesh floor. Each side contained a single, central light on the ceiling. The intensity of the light on each side was adjusted in preliminary experiments to balance the two sides as much as possible. However, to account for the baseline preference of each subject for one side of the chamber versus the other (Calcagnetti and Schechter, 1994; Prus et al., 2009), a biased CPP protocol was utilized, wherein the less preferred compartment was paired with the drug. During conditioning, each mouse received a saline injection in the morning (11:00 h) and was confined to the preferred compartment of the apparatus for 15 minutes. Each mouse received an ethanol injection in the afternoon (16:00 h) and was confined to the less preferred compartment for 15 minutes. Following the 4-day conditioning protocol, each mouse underwent a 15 minutes unrestricted access post-test to assess the effects of the conditioning. A dose effect curve of ethanol was performed in this manner to determine an appropriate dose of ethanol to use to test the effects of BCPO on the ethanol CPP. To assess the effects of BCPO on the development of the ethanol CPP, vehicle or BCPO (30 mg/kg) was administered 15 minutes prior to every ethanol injection during the 4-day conditioning protocol. The change in the time spent in the less preferred compartment was used as the dependent measure of a CPP.

2.6. Blood ethanol concentration (BEC)

Mice (N = 5 for each group) were injected with BCPO (30 mg/kg) or vehicle 15 minutes prior to the injection of ethanol (3.0 g/kg), which was the dose used in the LORR studies. At 1, 20, 60, 120 and 240 minutes after ethanol injection, blood samples were collected via the facial vein for each mouse as previously described (Golde et al., 2005). The sample was placed into prechilled BD Microtainer K_2EDTA tubes (Franklin Lakes, NJ) and immediately placed on ice. The pharmacokinetic profile of ethanol was assessed in the same subjects using a repeated-measures design. Blood was collected once per week for 5 weeks. The time point for blood collection was assessed in a randomized order as the time points were chose *a priori*. Blood samples were spun at 4200 rpm for 10 minutes at 4 °C after which 10ul of plasma from each sample was analyzed using Nicotinamide Adenine Dinucleotide-Alcohol Dehydrogenase (NAD-ADH) Reagent (Sigma-Aldrich St. Louis, MO) following the manufacturer's protocol. Briefly, NAD-ADH reagent was made up in 16ml glycine buffer, pH 9. 10ul of plasma samples was added to 1ml of NAD-ADH reagent in 1.5ml Eppendorf tubes and incubated at 25 °C for 10 minutes. A blank solution was made by adding 10ul of sterile water to 1ml NAD-ADH reagent. The solutions were transferred into cuvettes and the absorbance of the test solutions read at 339nm against the blank using a 2000C NanoDrop spectrophotometer (ND-2000C ThermoFisher, Waltham, MA).

2.7. Data analysis

All graphical data presentations were created and statistical analyses conducted using GraphPad Prism (La Jolla, CA). The LORR data were analyzed using one-way repeated-measures analysis of variance (ANOVA) with Dunnett's post-hoc test. Two-bottle choice and CPP data were assessed using paired t-test. The area under the curve (AUC) was determined for each mouse using the timecourse of its individual BEC. These AUCs were analyzed using unpaired t-test. Significance was arbitrated at $p < 0.05$.

3. Results

3.1. Comparative potency of BCP and BCPO in the LORR assay

To assess the applicability of a repeated-measures design to the study of the LORR, we first determined whether mice become tolerant or sensitized to the sedative effects of ethanol with once per week intraperitoneal injections. Changes in ethanol-induced loss of righting were assessed once per week (N = 7) for 6 weeks. One-way repeated-measures ANOVA revealed no significant difference in the LORR at any of the six time points (data not shown), suggesting that the mice neither became sensitized nor tolerant to the effects of ethanol using this dosing regimen, and supporting the use of a repeated-measures design for LORR experiments. We then assessed the effects of BCP and its derivative BCPO on the ethanol-induced LORR. These experiments were completed using the repeated-measures design and with an ascending dose order as described in the methods. The same animals were tested for all BCP doses and negative controls. Separate groups were tested for BCP and BCPO. The same animals were tested for all BCPO doses and negative controls. Each group completed the experiment within 6 weeks of repeated testing and all animals lost the righting reflex within the first 10 minutes after the ethanol injection in all weeks. We observed a significant main effect of dose for BCP ($F_{4,16} = 5.575$; $p = 0.005$) and BCPO ($F_{3,15} = 6.284$; $p = 0.006$) using one-way repeated-measures ANOVA. Dunnett's post-hoc analysis revealed that BCP at 300 mg/kg ($q = 4.282$) and BCPO at 30 mg/kg ($q = 4.011$) significantly increased sensitivity to the sedating effects of ethanol (Fig. 1). As such, the 30 mg/kg dose BCPO was utilized for all subsequent experiments. These findings demonstrate that BCPO is approximately 10 times more potent than BCP in modifying sensitivity to ethanol in this assay.

3.2. Two-bottle choice alcohol preference test

Mice were split into a high drinking group (N = 9) and a low drinking group (N = 9) following acquisition of stable ethanol drinking. The effects of BCPO were examined in both groups. In mice that consumed high amounts of ethanol, BCPO significantly reduced ethanol intake at 1 hour ($T_9 = 2.486$; $p < 0.05$) and 24 hours ($T_9 = 9.064$; $p < 0.001$) after provision of ethanol (Fig. 2). Paired t-test also showed that BCPO significantly reduced the preference for ethanol over water at 1 hour ($T_9 = 3.785$; $p < 0.001$) and 24 hours ($T_9 = 6.377$; $p < 0.001$) relative to vehicle. Demonstrating specificity to ethanol drinking, BCPO had no significant effect on total fluid intake in high drinking animals at 1 hour ($T_9 = 0.849$; $p = 0.421$) or 24 hours ($T_9 = 0.389$; $p = 0.707$). In animals that consumed low amounts of ethanol, paired t-test revealed that BCPO significantly reduced ethanol intake ($T_9 = 3.204$; $p = 0.013$) at 1 hour but had no such effect at 24 hours ($T_9 = 0.565$; $p = 0.588$) (Fig. 3). BCPO had no significant effect on ethanol preference at 1 hour ($T_9 = 1.250$; $p = 0.247$) or 24 hours ($T_9 = 0.393$; $p = 0.704$) or total fluid intake at 1 hour ($T_9 = 1.23$; $p = 0.253$) or 24 hours ($T_9 = 1.23$; $p = 0.253$) in animals that

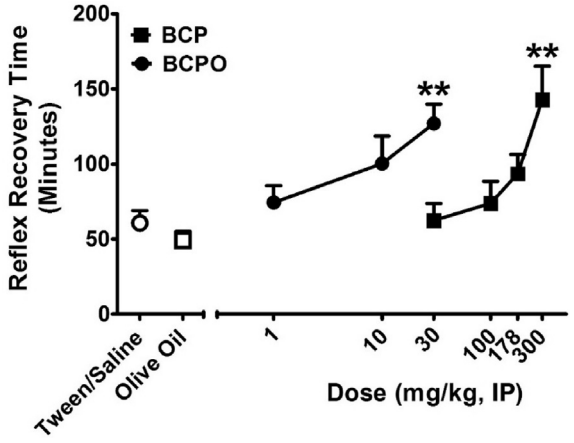

Fig. 1. Effects of β-caryophyllene (BCP) and β-caryophyllene oxide (BCPO) on the ethanol-induced loss of righting reflex. BCP significantly increased the reflex recovery time at a dose of 300 mg/kg whereas BCPO significantly increased the reflex recovery time at a dose of 30 mg/kg. *Abscissae*: Dosage of the drug treatment given before the injection of ethanol. *Ordinates*: Total time taken to recover the righting response following ethanol administration expressed in minutes. All values represent the mean +SEM. N = 5 for BCP, N = 6 for BCPO ** = $p < 0.01$ as assessed by one-way repeated-measures ANOVA in comparison to vehicle treatment.

consumed low amounts of ethanol.

3.3. Ethanol-induced place conditioning

An initial CPP dose-effect curve was established with ethanol at 1, 1.8, and 3 g/kg. The 1.8 g/kg dose of ethanol induced a significant CPP ($T_8 = 3.531$; $p = 0.009$) when analyzed by paired t-test in comparison to the pretest, and was chosen for further experiments with BCPO because it was the most effective dose in the dose response determination. Ethanol induced a significant CPP following daily treatment with the vehicle of BCPO prior to ethanol ($T_8 = 4.144$; $p = 0.004$), but not following daily treatment with BCPO prior to ethanol ($T_8 = 0.620$; $p = 0.552$) (Fig. 4).

3.4. Pharmacokinetic interactions

We hypothesized that BCPO may alter the metabolism or clearance of ethanol as we found that it increased the recovery time in the LORR study. As such, we next determined whether BCPO modified the pharmacokinetics of ethanol using blood-ethanol analysis. AUC analysis by unpaired t-test revealed no significant ($T_8 = 1.168$; $p = 0.281$) effect of BCPO in comparison to vehicle on this integration of the complete timecourse (out to 4 hours following administration) of blood ethanol levels following injection of a 3 g/kg bolus injection of ethanol (Fig. 5). These findings are not consistent with pharmacokinetic interactions between ethanol and BCPO.

3.5. Pharmacodynamic interactions

As we did not observe effects consistent with pharmacokinetic interactions between ethanol and BCPO, we then proceeded to examine whether there are pharmacodynamic interactions between BCPO and ethanol by antagonizing the CB2 receptor using the selective agent AM630. We targeted the CB2 receptor as it is known that the parent compound BCP stimulates CB2 receptors. We first assessed whether AM630 had any effect by itself on the ethanol-induced LORR in comparison to its vehicle. Administration of AM630 (1 and 3 mg/kg) alone prior to ethanol had no significant effect ($F_{2,23} = 3.471$; $p = 0.059$) on reflex recovery time. However, we did note that the effects of AM630 approached significance and that the 3 mg/kg dose appeared visually to have a stronger effect than the 1 mg/kg dose (Fig. 6, TOP). We then

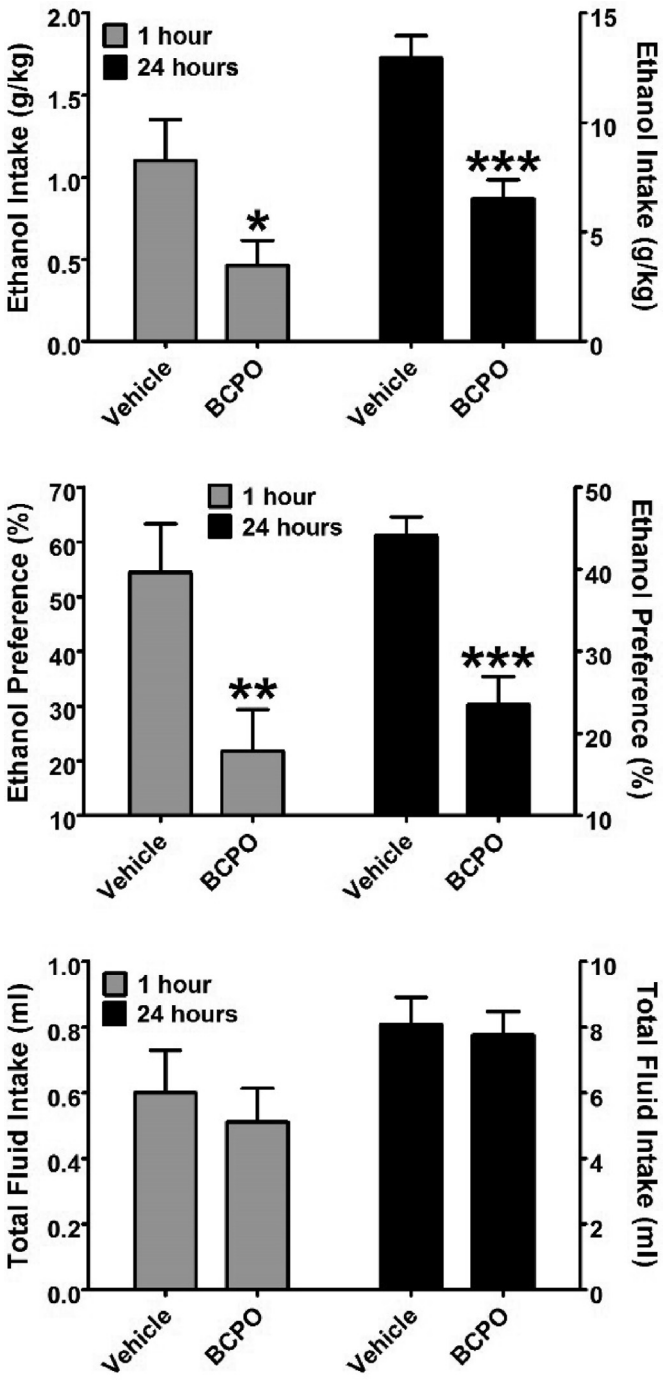

Fig. 2. Effects of a 30 mg/kg dose of β-caryophyllene oxide (BCPO) on ethanol consumption, ethanol preference, and total fluid intake at 1 hour and 24 hours in mice that stably consumed high amounts of ethanol. BCPO decreases ethanol consumption and preference at 1 hour and 24 hours in comparison to vehicle, without any effect on total fluid intake. TOP: Ethanol intake expressed in grams of ethanol per kilogram of body weight. MIDDLE: Preference for ethanol consumption over water consumption. BOTTOM: Total fluid intake expressed in milliliters of fluid. Data are subdivided to show the treatment effects 1 hour (gray column; left Y-Axis) and 24 hours (black column; right Y-Axis) after administration of vehicle or BCPO. All values represent the mean ± SEM. N = 9 * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ as assessed by paired t-test in comparison to vehicle treatment.

assessed whether AM630 attenuated the effects of BCPO using the 1 mg/kg dose of AM630. Pretreatment with AM630 significantly attenuated the LORR enhancing effects of the 30 mg/kg dose of BCPO ($T_8 = 4.801$; p =

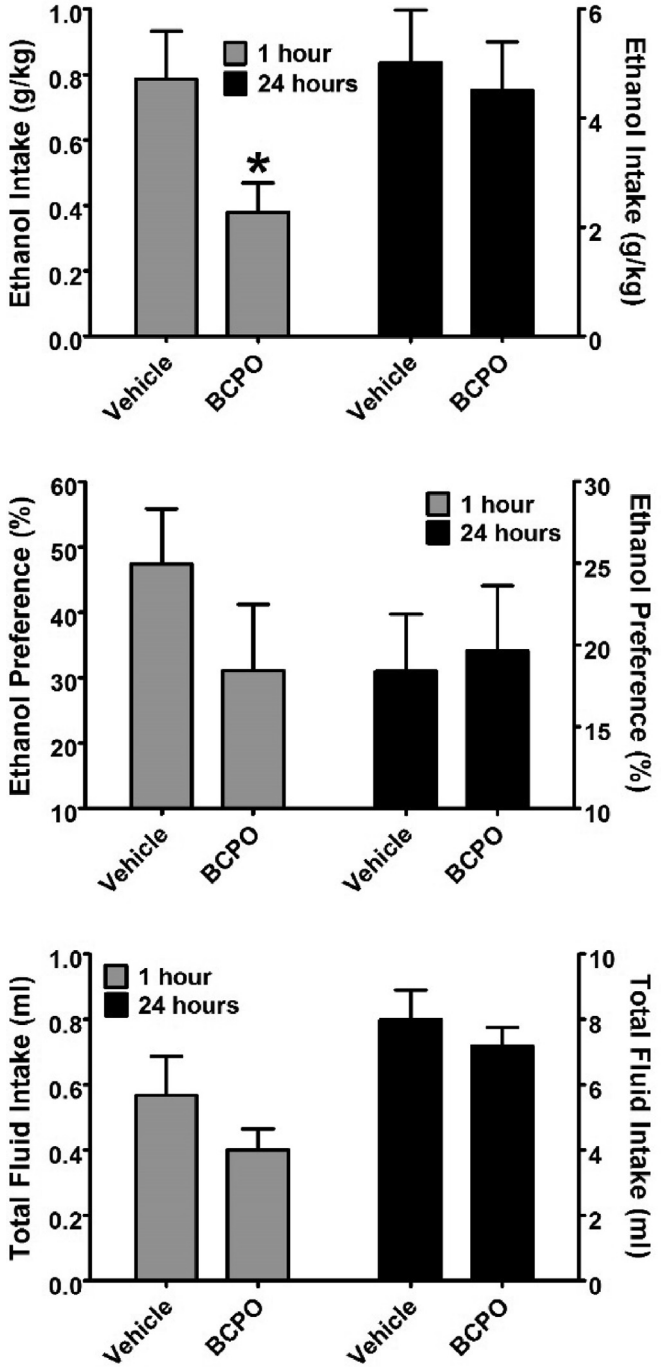

Fig. 3. Effects of a 30 mg/kg dose of β-caryophyllene oxide (BCPO) on ethanol consumption, ethanol preference, and total fluid intake at 1 hour and 24 hours in mice that stably consumed low amounts of ethanol. BCPO decreased ethanol consumption at 1 hour but not 24 hours, and had no significant effect on ethanol preference. TOP: Ethanol intake expressed in grams of ethanol per kilogram of body weight. MIDDLE: Preference for ethanol consumption over water consumption. BOTTOM: Total fluid intake expressed in milliliters of fluid. Data are subdivided to show the treatment effects 1 hour (gray column; left Y-Axis) and 24 hours (black column; right Y-Axis) after administration of vehicle or BCPO. All values represent the mean ± SEM. * = $p < 0.05$ as assessed by paired t-test in comparison to vehicle treatment.

0.001) in comparison to BCPO pretreated with the AM630 vehicle (Fig. 6, BOTTOM). This suggests that BCPO is capable of acting through CB2 receptors *in vivo*.

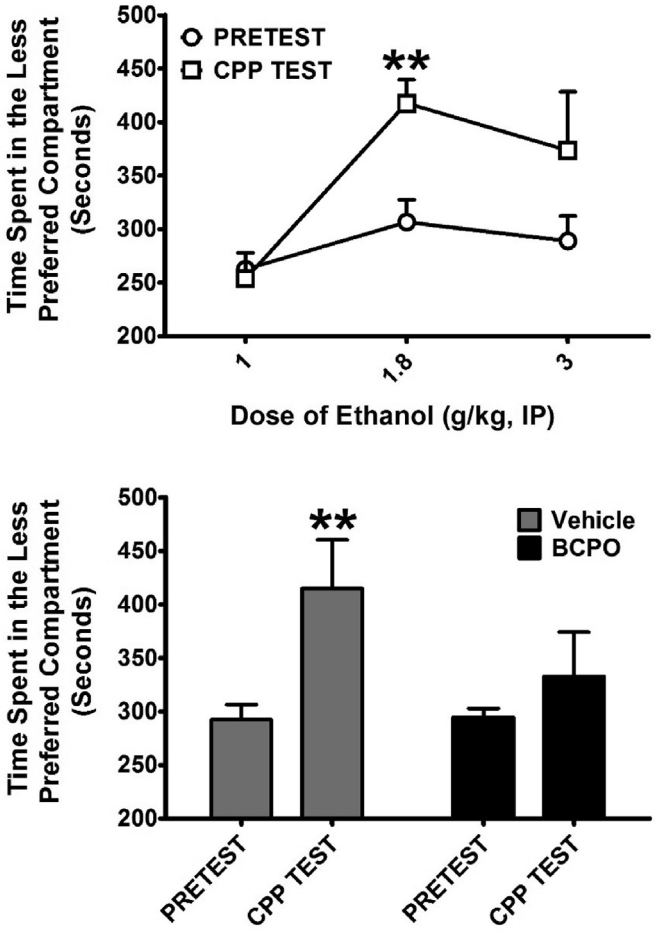

Fig. 4. TOP: Dose-effect function for the ethanol-induced conditioned place preference (CPP). BOTTOM: Effects of a 30 mg/kg dose of β-caryophyllene oxide (BCPO) on the ethanol-induced CPP. Data are expressed as time spent in the less preferred compartment (total time = 900 seconds) of the session time of 900 seconds. All values represent the mean +SEM. N = 8. ** = $p < 0.01$ in comparison to the pretest.

4. Discussion

The main aims of the study were to compare the potency of BCP and BCPO, determine whether BCPO attenuates ethanol drinking and place conditioning, and assess whether these effects of BCPO are more likely to be mediated by pharmacokinetic or pharmacodynamic interactions with ethanol. Our initial observation was that BCPO is 10 times more potent than BCP in increasing sensitivity to ethanol in the LORR assay. Using the dose of BCPO that was effective in the LORR assay (30 mg/kg), we found that BCPO decreased ethanol consumption and preference with no effect on total fluid intake in mice that consumed high amounts of ethanol. This is not likely a result of general suppression of drinking behavior as BCPO selectively reduced ethanol intake and preference with no effect on total fluid intake. In contrast to this, BCPO had no significant effect on 24-hour ethanol intake or ethanol preference in mice that consumed low levels of ethanol. BCPO did however significantly decreased ethanol intake in the first hour in mice that consumed low levels of ethanol. This indicates that there may be a distinguishable difference in the physiology or neurobiology of these animals that resulted in different responses to BCPO, and which could be explored to delineate the neuropharmacological and or neurobiological mechanisms involved. Ethanol affects both the innate and adaptive immune system, leading to increased production of proinflammatory cytokines and impaired production of anti-inflammatory

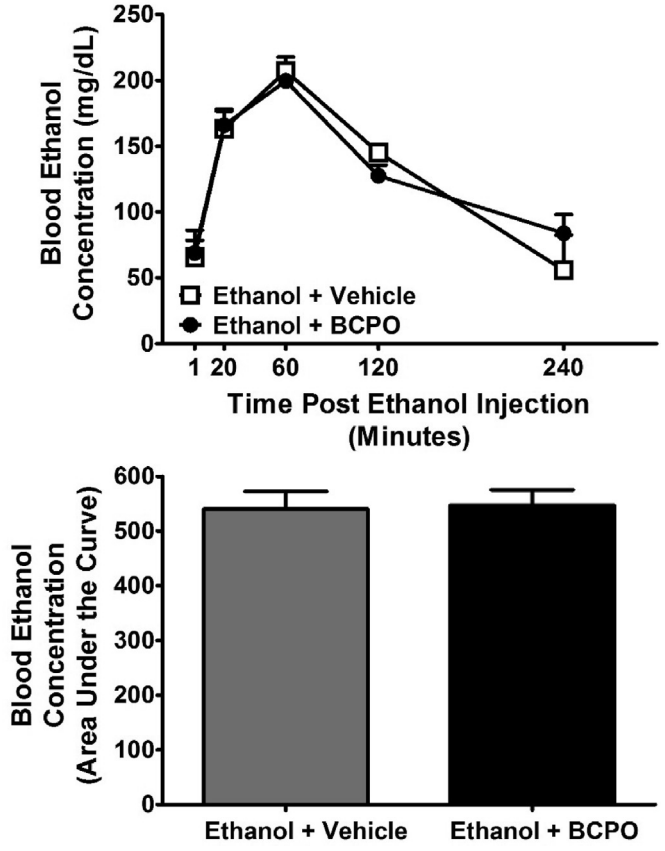

Fig. 5. Blood ethanol concentrations assessed over a period of 4 hours after a bolus injection of 3 g/kg of ethanol administered following either the vehicle of β-caryophyllene oxide (BCPO) or a 30 mg/kg dose of BCPO. BCPO did not significantly alter the timecourse of blood ethanol levels. TOP: Timecourse of ethanol concentrations in blood. BOTTOM: Area under the curve determined across integrations of the timecourses from individual mice. The x-axis is time is expressed in minutes following intraperitoneal ethanol administration (top) or treatment condition (bottom). Blood ethanol concentrations are expressed in mg/dl (top) or as the total integrated peak as assessed by the area under the curve (bottom). N = 5. Group differences in the area under the curve were assessed by unpaired t-test.

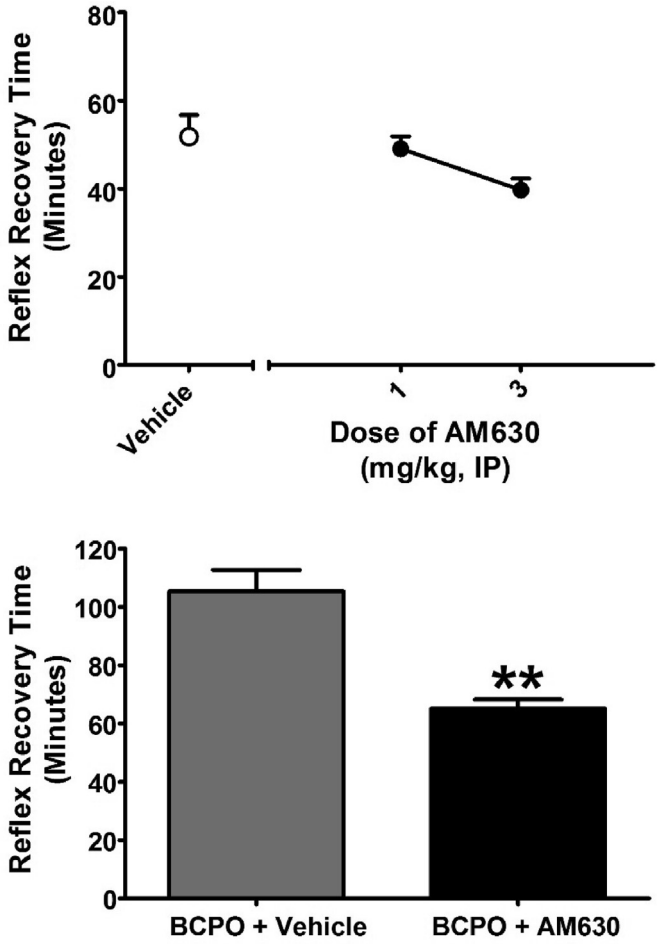

Fig. 6. TOP: Effects of the CB2 receptor antagonist AM630 administered alone on the ethanol-induced loss of righting reflex. N = 9. The data were assessed by one-way repeated-measures ANOVA in comparison to pretreatment of ethanol with the vehicle of AM630. BOTTOM: Augmentation of the ethanol-induced loss of righting reflex by β-caryophyllene oxide (BCPO) is significantly attenuated by a dose of AM630 (1 mg/kg) that had no effect by itself. N = 9 ** = $p < 0.01$ as assessed by paired t-test in comparison to BCPO given after administration of the vehicle of AM630. The total time taken to recover the righting response following ethanol administration is expressed in minutes. All values represent the mean +SEM.

cytokines (Szabo and Saha, 2015), which can result in alcohol-induced neuroinflammation (Lippai et al., 2013). BCPO has been shown to reduce nitric oxide production in macrophages stimulated by lipopolysaccharide, and thereby likely has anti-inflammatory effects (Tung et al., 2008). It is therefore possible, albeit speculative, that these anti-inflammatory effects may contribute to its enhanced efficacy in high drinking animals, as these subjects may be susceptible to greater neuroinflammation resulting from high intake of alcohol.

Our results are consistent with literature showing that phytomedicines have shown efficacy in reducing voluntary alcohol intake with no significant effect on total fluid intake. Lin et al. reported that the isoflavanoids decreased ethanol intake by 40–75% after oral administration. The effects of these isoflavanoids were specific for ethanol as there was no significant effect on total fluid intake. It also did not appear to be mediated by altering the pharmacokinetics of ethanol as the activity of liver alcohol dehydrogenase and aldehyde dehydrogenase were not affected after oral administration of these compounds (Lin et al., 1996). Human studies involving male and female heavy drinkers who were treated with root extracts of kudzu for seven days reported a significant decrease in alcohol consumption. These changes were observed in the absence of a significant effect on the urge to drink alcohol (Lukas et al., 2005). Phytocannabinoids have specifically garnered attention for their capacity to modulate ethanol drinking. As noted, Al Mansouri and colleagues reported a significant decrease in voluntary alcohol intake and attenuation of an ethanol CPP with BCP in mice (Al Mansouri et al., 2014). Our findings build upon this literature by showing that the phytocannabinoid BCPO also modulates the behavioral effects of ethanol, including ethanol drinking. This extends the case for further research with cannabinoids to mechanistically explore new targets and treatments for AUD. As alcohol is the most widely used psychoactive drug in the United States and the third leading cause of preventable death (Mokdad et al., 2004), this is an area of unmet need that would benefit from more research with phytocannabinoids.

An important finding of the study was that BCPO was 10 times more potent than BCP in increasing the sensitivity of mice to ethanol in the LORR assay, as evident in its leftward shift relative to BCP in the dose response function. Another major finding was BCPO had no significant effect on the timecourse of the BEC following acute treatment, suggesting that pharmacokinetic interactions between BCPO and ethanol do not account for its activity in the LORR assay. This also implies that BCPO has no interaction with ethanol metabolizing enzymes and has a reduced

likelihood of unexpected drug interactions with compounds metabolized by the same or similar enzymes. Moreover, the mechanism through which BCPO enhances the efficacy of ethanol in the LORR assay appears likely to be through stimulation of CB2 receptors, as its effects in the LORR assay were abrogated in the presence of AM630, a selective CB2 antagonist, at a dose of AM630 that did not affect the response to ethanol in the LORR assay by itself. These data are supportive of the idea that BCPO functions as a CB2 receptor agonist *in vivo*, similar to the parent compound BCP. Moreover, BCPO appears to potently act through CB2 receptor to modulate the sedating effects of ethanol. It may prove useful in elucidating interactions between the endocannabinoid system (ECS) and ethanol.

The ECS has been implicated in the rewarding effects of cannabinoids, nicotine, opioids and alcohol. As BCPO attenuated ethanol-induced place conditioning in addition to ethanol drinking, BCPO may be modifying the reinforcing and appetitive conditioned effects of ethanol. Although BCPO appears to act through CB2 receptors *in vivo* to enhance the sedating effects of ethanol, further research is necessary to establish whether activity at CB2 receptors modulates the reinforcing and appetitive conditioned effects of ethanol, which is something that we did not test in this study. It is also important to note that our results conflict with a previous report that BCPO does not bind to CB2 receptors *in vitro* and exerts its effects independent of the ECS (Gertsch et al., 2008). Moreover, the present study was conducted exclusively in male mice, and it is not yet clear whether the findings will generalize to females. Although further research is necessary to clarify these areas, the present study extends the literature and builds a solid foundation for this future research.

In conclusion, our study shows that BCPO is more potent in the *in vivo* LORR assay than BCP, and BCPO significantly attenuates voluntary ethanol consumption and an ethanol-induced CPP in mice. Our findings support the continued study of the potential for phytomedicine in the treatment of AUD and possibly other substance-use disorders, but warrant further studies into the precise mechanism of action of BCPO in alcohol dependence.

Declarations

Author contribution statement

Aboagyewaah Oppong-Damoah: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Bruce E. Blough, Alexandros Makriyannis: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Kevin Sean Murnane: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Funding statement

This work was supported by the National Institutes of Health [DA040907 and NS100512], and by funding from the Mercer University College of Pharmacy to KSM.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

These studies represent partial fulfillment of AOs PhD dissertation research project at Mercer University.

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