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Anxiolytic effect of an extract of *Salvia miltiorrhiza* Bunge (Danshen) in mice

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ABSTRACT

Ethnopharmacological relevance: Salvia miltiorrhiza Bunge (Danshen), a traditional Chinese medicine, has demonstrated in modern studies for its pharmacological activities in treatments of CNS disorders like insomnia, dysphoria. However, its application on anxiolytic effect from the ethanol extract of *Salvia miltiorrhiza* Bunge (SM_{EtOH}) has not yet been reported.

Materials and methods: This study investigated the anxiolytic effect of the SM_{EtOH} using the elevated plus-maze test (EPM) and the hole-board test (HBT) with diazepam and buspirone as positive controls. Also, the spontaneous locomotor activity of mice had been investigated in the open field. Further, we have illustrated the anxiolytic mechanisms of SM_{EtOH} with its influencing upon GABAergic and/or serotonergic nervous systems via a method that SM_{EtOH} was co-administered with flumazenil, a benzodiazepine (BZD) antagonist, or a drug (WAY-100635), a selective $5HT_{1A}$ receptor antagonist.

Results: In hole-board test, results presented that SM_{EtOH} increased head-dip counts and duration time. On the other hand, a decrease in spontaneous locomotor activity was observed. In the EPM test, SM_{EtOH} increased the percentage of open-arm entries and the percentage of time spent in open arms. However, when SM_{EtOH} co-administered with flumazenil or WAY-100635, the anxiolytic effect of SM_{EtOH} was significantly counteracted.

Conclusion: From these results, we can conclude that the anxiolytic mechanism of SM_{EtOH} is exerted through an activation of the BZD and $5HT_{1A}$ receptors.

1. Introduction

Anxiety disorder is a psychiatric illness that affects physical, emotional, and cognitive behaviors. The common clinical symptoms include palpitation, sweating, dry mouth, muscular tension, headache, and mental discomfort (Emilien et al., 2002).

In 1961, chlordiazepoxide, the first benzodiazepine (BZD) derivative synthesized by Leo Sternbach, substituted barbiturates and became the most commonly prescribed anxiolytic drug of the time (Kessler and Wang, 2008). Currently, approximately 40 BZD derivatives are commercially available. However, many clinical reports have reported that BZDs have common side effects, including somnolence, floppy muscles, and attention deficits. A long-term use of BDZs may even lead to dependence, memory impairment, and cognitive dysfunctions (Dell'osso and Lader, 2013; Kalachnik et al., 2002). Compared with BZDs, buspirone, another widely used drug for anxiety disorders, may not cause dependence. However, buspirone must be continued for 1–2 weeks for the most satisfactory effects; therefore, it cannot be used for treating short-term anxiety symptoms (Salazar et al., 2001). Although selective serotonin receptor inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) are currently the clear first-line agents due to a combination of efficacy and safety, and also treating co-morbid depression, BZD has its potent anti-anxiety efficacy (National Institute for Health and Care Excellence, 2019).

In clinical practice of traditional Chinese medicine, physicians select herbal medicines for anxiety disorders, such as the bark of *Albizia julibrissin* (Leguminosae), *Zizyphi Spinosi* Semen (Rhamnaceae), *Scutellaria baicalensis* Georgi (Lamiaceae), *Gastrodia elata* (Orchidaceae), and

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[1] Equal contribution to this article (the efforts to conduct experiments).

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Uncaria rhynchophylla (Rubiaceae) (Kim et al., 2004; Peng et al., 2000; Hui et al., 2002; Jung et al., 2006a, 2006b). Modern pharmacological studies have revealed that herbal medicines possess anxiolytic effects via the GABAergic or serotonergic nervous system (Liu et al., 2015a).

Danshen is the dried rhizome of *Salvia miltiorrhiza* Bunge (Labiatae). According to current use in literature, danshen is widely used for treating neurasthenic insomnia, dysphoria (Wang et al., 2018). In vitro, we found that dihydroisotanshinone I and tanshinone IIA are the bioactive compounds present in Danshen. Dihydroisotanshinone I improves survival of patients with advanced lung cancer and targeting the relationship between macrophages and lung cancer cells (Wu et al., 2017). The tanshinone IIA inhibits protein kinase C and acts as antitumor activities (Lv et al., 2018). Also, several constituents e.g. tanshinone I, tanshinone II, miltirone were isolated from *Salvia miltiorrhiza*, including alcohol-soluble diterpenoid quinones, water-soluble phenolic acids, and essential oil constituents (Tung et al., 2013; Shi et al., 2019). Latest studies have shown that *Salvia miltiorrhiza* can protect ischemic injury, including ischemic stroke, via its anti-oxidative effects (Qian et al., 2019; Zhang et al., 2018).

In the study of Liu et al., the essential oils extracts from *Salvia miltiorrhiza* performed anxiolytic effect (Liu et al., 2015b). However, the diterpene quinone compounds were absent in the volatile fraction, and the anxiolytic effects of the ethanol extract have not been investigated before. Therefore, we investigated, in this study, the ethanol extract of *Salvia miltiorrhiza* (SM_{EtOH}) for its putative anxiolytic effect using the elevated plus-maze test (EPM) and the hole-board test (HBT). Diazepam (1 mg/kg) and buspirone (2 mg/kg) were used as positive controls. Moreover, the spontaneous locomotor activity of mice in an open field had been investigated with SM_{EtOH}. In order to have insights into the mechanisms of anxiolytic effect, drugs (flumazenil and WAY-100635 with antagonism on BZD and $5HT_{1A}$ receptors, respectively) were each co-administrated with SM_{EtOH} to examine the actions of SM_{EtOH} ameliorating the anxiety either through the GABAergic or serotonergic nervous system.

2. Materials and methods

2.1. Plant and chemicals

The dried rhizome of *Salvia miltiorrhiza* Bunge (Danshen) was obtained from Chang Gung Memorial Hospital, Chiayi (Taiwan). Diazepam (10 mg/2 mL ampoule) was obtained from China Chemical & Pharmaceutical Co., Ltd (Taiwan). Buspirone, WAY-100635, and flumazenil were purchased from Sigma Chemical Co. (USA).

2.2. Preparation of SM_{EtOH}

The material Danshen (603 g) was macerated in 95% ethanol for 24 h and repeated four times. These filtrates were combined and concentrated under a reduced pressure at 40 °C using a vacuum rotary evaporator to obtain crude ethanol extract (SM_{EtOH}), and about 20 g of the resulting SM_{EtOH} (3.31% in yield) was stored at −20 °C before use. The material of Danshen extract (10 mg) used in this work was determined by HPLC (see supplement material) and shown to have 2.4 and 12 μg of dihydroisotanshinone I and tanshinone IIA which are corresponding to 0.02 and 0.12% in the content, respectively.

For application in the study tests, SM_{EtOH} was diluted to three concentrations (20, 100, and 500 mg/kg for animal experiments) in 10% Tween 20.

2.3. Animals

ICR male albino mice (weight 22–24 g, age 8–12 weeks) purchased from BioLASCO Taiwan Co., Ltd. were used for this study and housed at a laboratory animal center, Chang Gung Memorial Hospital, Chiayi, Taiwan. These mice were maintained at 23 °C ± 1 °C under a 12-hr light: dark cycle with ad libitum access to food and water. Six animals in each dosing group were taken in order to achieve the significance of scientific statistics. All experimental protocols and animal care guidelines were approved by the Animal Care Committee of Chang Gung Memorial Hospital with ethics approval number 2008082603.

2.4. Drug administration

To investigate the anxiolytic effect of *Salvia miltiorrhiza* in Hole Board test (HBT), **Elevated Plus-Maze (EPM) test** and Spontaneous Locomotor Activity test, SM_{EtOH} (20, 100, and 500 mg/kg, p.o.) were administered to mice in comparison to a negative control (0.9% saline, p.o.). The trial was carried out at 60 min after SM_{EtOH} treatments. For positive controls, diazepam group (1 mg/kg, i.p.) and buspirone group (2 mg/kg, i.p.), were administered 30 min before the trial.

To further examine the mechanism of SM_{EtOH} in the EPM test, SM_{EtOH} (100 mg/kg, p.o.) and the control (0.9% saline, p.o.) were administered 60 min before the test. Doses of SM_{EtOH} were chosen based on others' studies and modified into this study (Cui et al., 2011; Chen et al., 2012; Qiao et al., 2011). Flumazenil (3 mg/kg, i.p.) or WAY-100635 (0.3 mg/kg, i.p.) alone was administered to mice 30 min before the trial as positive controls. Also, flumazenil (3 mg/kg, i.p.) or WAY-100635 (0.3 mg/kg, i.p.) was in combination with SM_{EtOH} 30 min after SM_{EtOH} (100 mg/kg, p.o.) was applied (see Table 2).

Each group included 6 mice. The study design and procedures were approved by the committee on Animal Care and Use in our hospital (No.2008082603).

2.5. Hole-board test (HBT)

The HBT is a method established by Boissier and Simon in 1962, and has been used to assess emotionality, anxiety and responses to stress in animals (Takeda et al., 1998). The hole-board apparatus comprises gray Perspex panels (40 cm × 40 cm × 30 cm) with sixteen 3-cm-diameter equidistant holes on the floor. The HBT was measured and analyzed by a computer and TruScan TV photobeam sensor E63-12 that have high-precision infrared light beam frames surrounded the housing (Coulbourn Instruments, U.S.). The mice were individually placed in the center of the experimental box. When the mouse sticks its head into the plate hole and blocks infrared rays, the number of times and the duration (seconds) were automatically recorded in total 5 min. The board was cleaned after each animal was tested.

2.6. Elevated plus-maze test (EPM test)

The EPM test was first designed by Montgomery in 1955 and is based on the animal anxiety model driven by their strong fear in an open space (Pellow et al., 1985). The EPM test for mice comprises two perpendicular open arms (30 cm × 5 cm) and two enclosed arms (30 cm × 5 cm) with 15-cm-high walls extending from the central platform (5 cm × 5 cm). The open and closed arms were connected by a 5 cm × 5 cm central square, thus presenting a "+ sign" appearance. The EPM test was measured and analyzed using the video based Ethovision System. The mice were placed in the center of the square. After continuous recording for 5 min, the count and duration of mice entering and staying in the open and closed arms were recorded. For a mouse with anxiety, a decrease of time spent in the open arm and open arm entries would be observed. By contrast, an increase of time spent in the closed arm and closed arm entries would be indicated for a mouse with anxiety. The EPM was cleaned after each animal was tested.

2.7. Spontaneous locomotor activity

Spontaneous locomotor activity was measured by using the TruScan TV photobeam sensor system E63-12 that have high-precision infrared light beam frames surrounded the housing (Coulbourn Instruments,

USA). This instrument comprised a 40 cm × 40 cm × 30 cm box in which the mice were placed for 5 min for allowing them to adapt to the new surroundings. Subsequently, their movements were continuously recorded for 5 min, and the total travel distance and the rest time were evaluated. For a mouse with sedation, a decrease in locomotor activity would be observed. This instrument was cleaned after each animal was tested.

2.8. Statistics

Each test includes 6 groups (6 mice per group). All results are expressed as mean ± standard error of the mean. Data were analyzed using one-way ANOVA and Scheffe's test. Differences between the experimental groups were considered significant when $p < 0.05$. All statistical analyses were carried out by using SPSS for Windows (SPSS Inc.).

3. Results

3.1. Effects of SM_{EtOH} on the hole-board test (HBT)

HBT has been used to assess emotionality, anxiety and responses to stress in animals. There are studies indicated that treatment with anxiolytic drugs such as diazepam and buspirone would affect exploratory behavior including increase head-dip counts and head-dip duration (Takeda et al., 1998; Pokk and Zharkovsky, 1998). Our study showed that compared with the control group (0.9% saline), diazepam and buspirone significantly increased the head-dip counts (Fig. 1A $p < 0.001$ and $p < 0.01$, respectively) and the head-dip duration (Fig. 1B $p < 0.001$ and $p < 0.01$, respectively) during the 5-min test in the mice. With similarities, SM_{EtOH} (100 and 500 mg/kg) caused an increase in the head-dip counts (Fig. 1A $p < 0.01$ and $p < 0.05$, respectively) and the head-dip duration (Fig. 1B $p < 0.01$ and $p < 0.05$, respectively). This indicates that SM_{EtOH} bears anxiolytic bioactivities as the clinical commercial drugs.

3.2. Effects of SM_{EtOH} on the **elevated plus-maze test** (EPM test)

EPM test is based on the premise that the exposure on an EPM evokes an approach-avoidance conflict. This conflict is considerably stronger than that evoked by an entry into enclosed arms. For mice, a decreased aversion to the open arms as a result of anxiolytic-like effect. The strength of anxiolytic effect is expressed by an increased number of open arm entries and time spent, or a decreased number of closed arm entries and time spent in the EPM (Pellow et al., 1985).

As shown in Fig. 2, these mice of the control group typically avoid spending their time on or entering into the open arms. Compared with the control group, the mice of the positive control group using diazepam or buspirone significantly increased the percentage of their entries and time spent on open-arms (Fig. 2A and B, respectively), but reduced the time spent on closed arms (Fig. 2B). Moreover, for the mice of SM_{EtOH} (100 mg/kg)-treated group, a significantly increase in the percentage of open-arm entries (Fig. 2A $p < 0.05$) was observed. Also, SM_{EtOH} (100 and 500 mg/kg) significantly increased their time spent on open arms but reduced the time spent on closed arms (Fig. 2B $p < 0.01$ and $p < 0.05$, respectively). Surprisingly, in Fig. 2A, the mice of SM_{EtOH} (500 mg/kg)-treated group did not showed a significantly increase in the percentage of open-arm entries. The possible explanation is elucidated with the following study of spontaneous locomotor activity.

3.3. Effects of SM_{EtOH} on the spontaneous locomotor activity

As shown in Table 1, compared to the control, SM_{EtOH} with 20 and 100 mg/kg did not affect total travel distances or rest time in the spontaneous locomotor activity experiments. However, SM_{EtOH} at 500 mg/kg showed a reduction of total travel distances as well as rest time in the mice significantly ($p < 0.01$ and $p < 0.05$).

3.4. Anxiolytic activity of SM_{EtOH} through affecting on GABAergic or serotonergic paths

In order to understand the anxiolytic mechanism of SM_{EtOH} either via GABAergic or serotonergic transmission, SM_{EtOH} (100 mg/kg) was co-administered with flumazenil (a BZD antagonist) and WAY-100635 (a $5HT_{1A}$ receptor antagonist). As shown in Table 2, compared to the control, SM_{EtOH} (100 mg/kg) significantly increased the percentage of open-arm entries and time spent, and reduced the time spent in closed arms ($p < 0.01$). Moreover, the use of flumazenil and WAY-100635 exerted an anxiogenic (causing anxiety) effect and showed no significant difference to the control. However, in comparison to SM_{EtOH}, groups of flumazenil or WAY100635 co-administrated with SM_{EtOH} (100 mg/kg) remarkably decreased the percentage of open-arm entries and time spent on open arms, as well as increased time spent on closed arms. Also, the time spent of SM_{EtOH} (100 mg/kg) treated-mice in closed arms was much shorter than the control and than mice treated with flumazenil and WAY 100635 alone or together with SM_{EtOH} (100 mg/kg) (Table 2). This means that the anxiolytic effect of SM_{EtOH} was significantly counteracted in presence of flumazenil and WAY-100635. Therefore, the anxiolytic activity of SM_{EtOH} is through affecting on the transmission of BZD and $5HT_{1A}$ receptors.

4. Discussion

The hole-board test (HBT) and e**levated plus-maze** (EPM) test were used in this study for evaluating animal anxiety caused by the conflict between environmental stimuli and the exploratory nature of mice. To administer diazepam or buspirone can produce anxiolytic effects and alter the anxiety behaviors in HBT and EPM test (Takeda et al., 1998; Pellow et al., 1985; Pokk and Zharkovsky, 1998). In the HBT, the mice treated with 100 mg/kg SM_{EtOH} showed a significantly increase in their

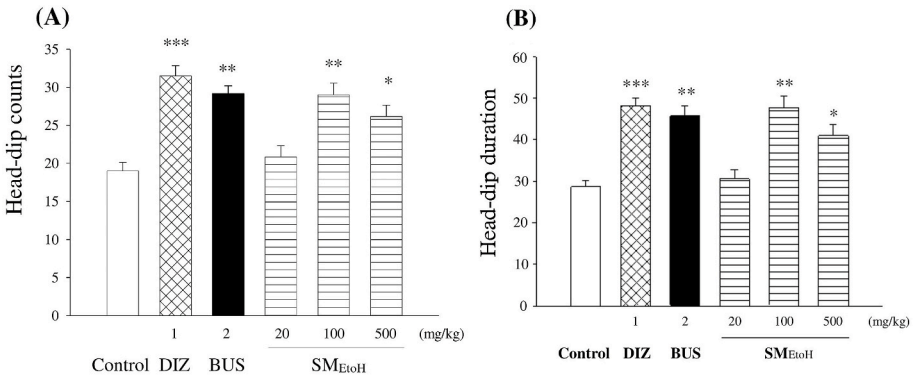

Fig. 1. Effects of diazepam (DIZ, 1 mg/kg), buspirone (BUS, 2 mg/kg), and SM_{EtOH} on (A) head-dip counts and (B) head-dip duration of the mice in the hole-board test during a 5-min test. Data are presented as mean ± standard error of the mean (n = 6). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ in comparison with the control group (normal saline) (one-way ANOVA, degree of freedom (df) of between groups and within groups are 5 and 30, followed by Scheffe's test). The mean difference is significant at the 0.05 level.

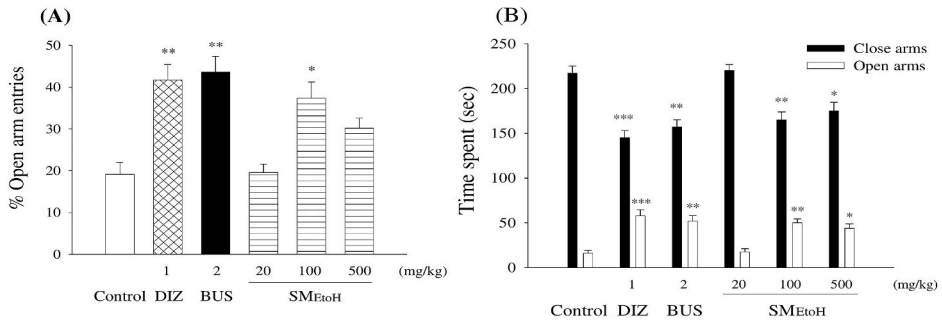

Fig. 2. Effects of diazepam (DIZ, 1 mg/kg), buspirone (BUS, 2 mg/kg), and SM_{EtOH} on (A) percentage of open-arm entries and (B) time spent in the open and closed arms of the elevated plus-maze. Data are presented as mean ± standard error of the mean (n = 10). $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared with the control group (one-way ANOVA, df of between groups and within groups are 5 and 30, followed by Scheffe's test). The mean difference is significant at the 0.05 level.

Table 1
Effects of SM_{EtOH} on the changes in total travel distance and rest time of the mice. Data are presented as mean ± SEM (n = 6). $*p < 0.05$ and $**p < 0.01$ compared to the control (normal saline) (one-way ANOVA, df of between groups and within groups are 5 and 30, followed by Scheffe's test). The mean difference is significant at the 0.05 level.

Treatment (dose: mg/kg)	Total travel distance (cm)	Rest time (sec)
Control	458.10 ± 14.65	69.40 ± 5.30
SM_{EtOH} (20 mg/kg)	423.83 ± 20.18	67.20 ± 4.39
SM_{EtOH} (100 mg/kg)	437.56 ± 25.93	70.00 ± 5.07
SM_{EtOH} (500 mg/kg)	319.66 ± 16.70**	97.33 ± 7.55*

Table 2
Anxiolytic effect of SM_{EtOH} and its interaction with drugs antagonizing BZD and $5\text{-}HT_{1A}$ receptors in the elevated plus-maze test. The values are expressed as mean ± SEM (n = 10).$^{\#}$ $^{\#}p < 0.01$, compared to the control (normal saline). $*p < 0.05$, $**p < 0.01$, compared to SM_{EtOH} (100 mg/kg) group (one-way ANOVA, df of between groups and within groups are 5 and 30, followed by Scheffe's test). The mean difference is significant at the 0.05 level.

Treatment (mg/kg)	Open arms entries (%)	Time spent in open arms (sec.)	Time spent in close arms (sec.)
Control	19.67 ± 2.48	17.50 ± 2.86	218.16 ± 8.96
SM_{EtOH} (100 mg/kg)	38.70 ± 3.48$^{\#\#}$	46.50 ± 5.66$^{\#\#}$	166.66 ± 5.83$^{\#\#}$
Flumazenil (3 mg/kg)	16.68 ± 3.59	16.33 ± 3.81	216.16 ± 8.56
Flumazenil + SM_{EtOH}(100 mg/kg)	19.36 ± 2.13 **	23.16 ± 4.68 *	213.83 ± 7.79 *
WAY 100635 (0.3 mg/kg)	17.05 ± 1.25	17.66 ± 3.59	224.33 ± 9.71
WAY 100635+ SM_{EtOH}(100 mg/kg)	21.82 ± 3.93*	22.00 ± 3.24*	208.16 ± 6.31*

head-dip counts and duration. In the EPM test, 100 mg/kg SM_{EtOH} significantly increased the percentage of open-arm entries and the time spent on the open arms and reduced the time spent on the closed arms. In 2018, a study showed that intra-gastrically administering 100 mg/kg *Salvia miltiorrhiza* ethanol-extract produced an equal anxiolytic effect as 1.5 mg/kg (i.p.) of the reference compound, diazepam in Wistar rats (Lobina et al., 2018). In our study in mice, SM_{EtOH} (100 mg/kg, p.o.) produced similar anxiolytic effects as diazepam (1 mg/kg) and buspirone (2 mg/kg), these commercial drugs were given via i.p. route. It is reasonable because parenteral-use diazepam has been shown to be more effective in reducing anxiety and stress as compared with an equivalent oral dose (Browning et al., 1987). Nevertheless, additional information is that the components of SM_{EtOH} via p.o. route could be also as effective in applications on suppressing anxiety as diazepam and buspirone.

Salvia miltiorrhiza belongs to the genus *Salvia* that includes many plants exhibiting CNS sedative activity and been used for treating CNS diseases (Imanshahidi and Hossein, 2006). Among them, ethanol extracts of *S. guaranitica* and *S. haematodes* also have sedative effects and prolong the pentobarbital-induced sleep duration (Imanshahidi and Hossein, 2006). Hydroalcoholic extracts of *S. elegans* and *S. reuterana* Boiss demonstrated anxiolytic and sedative activity in mice (Rabbani et al., 2005; Herrera-Ruiz et al., 2006). When in the spontaneous locomotor activity test, the mice treated with 500 mg/kg SM_{EtOH} reduced the total travel distance and increased their rest time. This is evident that SM_{EtOH} can demonstrate not only axiolytic effects but also sedative effects. Also, 100 mg/kg SM_{EtOH} did not show significant sedative effects but only have anxiolytic effect. The reasonable explanation is that anxiolytic effects and sedative effects are dependent to the dose (Uvnäs-Moberg et al., 1994). This observation is consistent with the guidelines of tradition Chinese medicine prescription, which mentions that nourishing the heart and tranquilizing the mind requires a low dose of Danshen, whereas sedation requires a high dose (Fang et al., 2010).

Several studies suggest that GABAergic and serotonergic systems would be involved in the anxiolytic-like effects (Peng et al., 2004). In our study, flumazenil, a BZD antagonist and WAY100635, a selective $5\text{-}HT_{1A}$ receptor antagonist, can reverse an anxiolytic-like effect when SM_{EtOH} was co-administered in the EPM test. This is an indication that the presence of SM_{EtOH} would affect both GABAergic and serotonergic systems and the anxiolytic effect of SM_{EtOH} may be due to activation of the BZD and $5HT_{1A}$ receptors.

5. Conclusion

In summary, the results of our study demonstrated that SM_{EtOH} possessed anxiolytic effects in HBT and EPM test in mice. Also, the study result is coherent with the prescription in tradition Chinese medicine literature. For its development as an anti-anxiety drug, SM_{EtOH} shall be further studied for its effectiveness (on anti-anxiety) and safety assessment (on sedation) in clinical trials.

Declaration of competing interest

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2020.113285.

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