Anxiety and depression are debilitating, costly psychological disorders that account for $133 billion in direct medical expenses per year in the United States. Finding alternative means of treatment to reduce the personal and financial burden for patients with these disorders, while maintaining patient safety, is vital for overall patient wellness. The purposes of this study were 2-fold: (1) to determine if pure eucalyptol (1,8-cineole) produces anxiolytic and/or antidepressant effects using rat models for anxiety and behavioral despair and (2) to determine the effects of eucalyptol at the benzodiazepine site on the γ-aminobutyric acid (GABA) A receptor in the rat central nervous system. Fifty-five male Sprague-Dawley rats were randomly assigned to 1 of 5 groups: vehicle (dimethyl sulfoxide), eucalyptol, midazolam, flumazenil plus eucalyptol, and midazolam plus eucalyptol. Behavioral analyses were conducted on the elevated plus-maze and in the forced swim test. Data were analyzed using a 2-tailed multivariate analysis of variance and a least significant difference post hoc test.

Data from the maze suggested eucalyptol may produce anxiolytic effects by acting at the benzodiazepine site on the GABA A receptor while not affecting psychomotor activity. However, no effects on behavioral despair were demonstrated in the Forced Swim test.

Keywords: Anxiolysis, elevated plus-maze, eucalyptol, eucalyptus, Sprague-Dawley rat.
Factors motivating US adults to take herbal supplements have historically been unidentified. In 2013, data from the National Health and Nutrition Examination Survey, a cross-sectional, population-based survey (n = 11,956), was published and determined specific reasons that adults used herbal supplements. The 2 largest motivating factors identified for herbal supplement use were “improving overall health” and “maintaining health,” which accounted for 45% and 33% of those surveyed, respectively. Four percent of the surveyed adults also reported “mental health” as a motivating factor for using herbal supplements. In 2004, McPherson and Schwenka reported rates of herbal supplement use as high as 30% among beneficiaries of the US military healthcare system, with the primary motivational reasons being management of pain, stress, anxiety, and depression.

Despite significant use of herbal supplements throughout civilian and military populations, side effects of these compounds remain largely unknown. Understanding the side effects, interactions, and toxicity of herbal supplements is an important aspect of patient safety. Many patients self-medicate with herbal supplements to treat anxiety and/or depression, unaware of potentially dangerous pharmaceutical interactions. The combined effects of herbal supplements with prescribed pharmaceuticals is such an important patient safety issue that The Joint Commission mandates that all medications and herbal supplements taken by patients be reviewed for possible interactions. For healthcare providers to safely prescribe pharmaceutical treatments, it is imperative that they are aware of synergism, interactions, and/or antagonism of prescribed medications with herbal supplements.

One of the many herbal supplements found on the market today is eucalyptus oil, which is extracted from the leaf of the eucalyptus tree. Eucalyptus oil has been used for centuries and studied for a variety of medicinal benefits as a food preservative, antibacterial and antiviral agent, anti-inflammatory agent, and a treatment of asthma. The active compound found in eucalyptus oil is eucalyptol, also known as 1,8-cineole, which is composed of as much as 70% of eucalyptus oil.

Emerging research on the effects of eucalyptol as an anxiolytic and antidepressant suggests promising results in both human and animal models. Kim and colleagues found that inhaled eucalyptus oil significantly reduced preoperative anxiety in humans undergoing selective nerve root block procedures. In rodent models, anxiolytic and antidepressant effects were found after the administration of essential oils containing various percentages of eucalyptol. These results support the research by Kaewwongse et al, in which cineole (eucalyptol) demonstrated anxiolytic effects on male rats subjected to the elevated plus-maze (EPM) and an open-field test. Kaewwongse et al suggested the anxiolytic effects of cineole (eucalyptol) are related to flavonoids, which are structurally similar to diazepam, a benzodiazepine, and would therefore modulate the benzodiazepine site on the GABA receptor. Machado et al found antidepressant effects in mice using the Tail Suspension test after administering a compound containing 45% eucalyptol. Quílez and colleagues investigated spontaneous motor activity of mice when given essential oil containing eucalyptus (1.5%) and diazepam. This study demonstrated that eucalyptus decreased the action of diazepam on the muscles and spontaneous movement of mice, thereby indicating that they may both compete for the same binding site on the GABA receptor. Conversely, flumazenil, a benzodiazepine-receptor antagonist, which reverses the effects of benzodiazepines in the central nervous system, was not shown to affect anxiety in rat models when administered in conjunction with compounds containing eucalyptol (21%). However, these studies used essential oils containing other potentially active compounds instead of pure eucalyptol, which may explain some of the observed effects. All previously listed studies have called for further research of eucalyptol and its ability to demonstrate anxiolytic and/or antidepressant effects.

Patients undergoing surgical procedures often experience anxiety, which results in a physiologic stress response promoting immunosuppression, higher rates of infection, elevated blood glucose levels, and even delayed wound healing. Anesthesia providers reduce anxiety with the administration of anxiolytic medications such as benzodiazepines. Midazolam, a water-soluble benzodiazepine, has been shown to reduce anxiety in multiple studies. It is unknown whether the administration of eucalyptol exerts either anxiolytic or antidepressant effects by the modulation of the benzodiazepine site on the GABA receptor. Therefore, the purposes of this study were to determine whether eucalyptol produces anxiolytic and/or antidepressant effects using rat models that evaluate anxiety and behavioral despair, and to examine the effects of eucalyptol at the benzodiazepine site on the GABA receptor in the rat central nervous system.

Materials and Methods

• Animals. Fifty-five male Sprague-Dawley rats, each weighing between 250 and 300 g, were obtained commercially (Envigo RMS Inc, formerly Harlan Laboratories Inc.). Rats were housed in groups of 3, in clear plastic rodent containers, with 109.6 to 451.5 cm (17 to 70 sq in) of floor space per rat containing appropriate bedding material and environmental enrichment. Over a 14-day period, light and dark cycles were used to simulate circadian rhythms in a controlled environment (22 ± 1°C and 60% humidity). The light cycle was between 12:01 am and noon, and the dark cycle was between 12:01 pm and midnight. Water and food were provided ad libitum.

To prevent any confounding variables, the same investigator performed all gentling of the rodents over the
14-day experimental period: cage cleaning, obtaining daily weights, drug administration, and movements of rats to and from testing procedures. All experiments were conducted in accordance with the Institutional Animal Care and Use Committee at the US Army Institute of Surgical Research, Joint Base San Antonio–Fort Sam Houston, Texas, and according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals guidelines.

- **Drugs.** Eucalyptol (99%), flumazenil (> 99%), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Midazolam was obtained from the US Army Institute of Surgical Research pharmacy and met pharmaceutical-grade standards. A 0.5% concentration of DMSO was used as the solvent for flumazenil, and normal saline was used as the diluent for eucalyptol and midazolam.

- **Experimental Design.** Fifty-five rats were randomly assigned to 1 of 5 groups (n = 11 per group). Each of the 5 groups received 2 intraperitoneal injections as follows: group 1: DMSO plus normal saline; group 2: DMSO plus eucalyptol (1 mg/kg); group 3: DMSO plus midazolam (1.5 mg/kg); group 4: flumazenil (3 mg/kg) plus eucalyptol (1 mg/kg); and group 5: midazolam (1.5 mg/kg) plus eucalyptol (1 mg/kg). Midazolam, flumazenil, and eucalyptol solutions were prepared fresh each day. All drugs were administered using 1-mL latex-free syringes with a 25-gauge, 1.27-cm (0.5-in) needle 30 minutes before the first test. Group 1 was used as the negative control group. Group 2 was used to assess for anxiolytic and/or antidepressant effects of eucalyptol. Group 3 was used as the positive control group. Group 4 was used to determine any effects seen in the eucalyptol group that could be attributed to the benzodiazepine binding site of the GABA<sub>A</sub> receptor; the flumazenil was given 10 minutes before the eucalyptol. Group 5 was used to assess for any interactions between midazolam and eucalyptol.

- **Elevated Plus-Maze.** The EPM is a valid and reliable assessment tool for anxiety in the rat model. Increased open-arm time exploration indicates decreased anxiety. The EPM consists of 2 open arms and 2 closed arms, with each arm measuring 50 cm × 10 cm. The closed arms had walls that spanned the length of the arm and did not have a roof. The open arms were positioned perpendicular to the closed arms. The maze was elevated to a height of 50 cm to prevent the rats from escaping (Figure 1). All rats were assessed on the EPM for 5 minutes. The open-arm time ratio is the percentage of time spent in the open arm. Behavioral testing on the EPM lasted 5 minutes. Photograph displays a rat in the open arm with an orange tracking dot projected by the tracking software (AnyMaze, San Diego Instruments).

- **Forced Swim Test (FST).** The FST was used to assess data on mean time mobile (seconds) and fecal pellet output. Time mobile was defined as the rat exhibiting movements necessary to keep its head above water for 5 minutes. Cylinders made of plexiglass measured 20 cm in diameter × 40 cm high. Depth of the water prevented the rat from touching the bottom of the cylinder and was maintained at a constant temperature of 25 ± 2°C.
were injected with study medications 30 minutes before the behavioral test on the EPM. Following the injections, the rats were placed back into their housing unit until the start time of the EPM. All movements were recorded with a video recorder placed directly above the center of the EPM, and data were digitalized using tracking software (AnyMaze, San Diego Instruments). Thirty-seven individual data points were recorded, such as number of entries into the closed or open arms, distance traveled, and time spent mobile or immobile. Mean time mobile was recorded in meters per second and then converted to centimeters per second. Open-arm time ratio was calculated by taking the time spent in the open arm divided by the total time and multiplying by 100 to get a percentage. The EPM test lasted 5 minutes, at which point it was cleaned with soap and water and allowed to dry between experiments.

**Forced Swim Test.** The FST is a valid tool in the assessment of behavioral despair in the rodent model. Following the EPM, rats were carried by the investigator to a separate room for evaluation in the FST. Each rat was placed into an individual plexiglass cylinder measuring 20 cm in diameter and 40 cm high (Figure 2). The water was maintained at 25 ± 2°C and at a depth sufficient to prevent the rat from touching the bottom of the cylinder. After each video-recorded FST test, the water was changed. Twenty-four hours before testing, a Forced Swim test habituation session lasting 15 minutes was conducted with each rat. Habituation acclimated the rats to the test, providing a reliable level of immobility behavior during the 5-minute test 24 hours later. The investigators counted fecal pellet output (FPO) after each experiment during the water change. Two investigators, blinded to treatment groups, measured time mobile and FPO. For the purposes of this experiment, time mobile was recorded as any time other than when the rat is passively floating and exhibiting movements necessary to keep the head above water. Decreased time mobile reflected behavioral despair, and increased FPO indicated increased stress.

**Statistical Analysis.** Data were collected from 55 rats; all data were analyzed using a 2-tailed multivariate analysis of variance and a least significant difference (LSD) post hoc test.

**Results**

The rats weighed 255 to 319 g, without statistically significant differences between the groups (Table 1).

**Elevated Plus-Maze.** The EPM was used to evaluate the following variables: mean speed (centimeters per second), mean time mobile (seconds), and open-arm time ratio (percentage). Mean speed in the eucalyptol group was significantly increased compared with that in the midazolam group ($P = .045$). The midazolam group
was significantly decreased in mean speed compared with all other groups: vehicle (P = .001), eucalyptol (P = .045), flumazenil plus eucalyptol (P = .049), and midazolam plus eucalyptol (P = .016). No other statistically significant differences were found between the groups regarding mean speed (Table 1, Figure 3A).

For mean time mobile, the eucalyptol group was significantly increased compared with the midazolam group (P = .001). The midazolam group was significantly decreased in mean time mobile compared with all other groups: vehicle (P = .001), eucalyptol (P = .021), flumazenil plus eucalyptol (P = .034), and the midazolam plus eucalyptol (P = .016). No other statistically significant differences were found between the groups regarding mean time mobile (Table 1, Figure 3B).

For open-arm time ratio, there was no significant difference when the eucalyptol group was compared with any other group. There was a significant increase in the midazolam group for open-arm time compared with the vehicle group (P = .005) and the flumazenil plus eucalyptol group (P = .03). There was also a significant increase in the midazolam plus eucalyptol group compared with the vehicle group (P = .007) and the flumazenil plus eucalyptol (P = .038; Table 1, Figure 3C).

- **Forced Swim Test.** The FST was used to evaluate mean time mobile (seconds) and FPO. There was no significant difference in mean time mobile between any groups on this test (Table 2, Figure 4).

Mean FPO was significantly increased in the eucalyptol group compared with the midazolam group (P = .032) and the midazolam plus eucalyptol group (P = .007); the flumazenil plus eucalyptol group was significantly increased compared with the midazolam group (P = .046) and the midazolam plus eucalyptol group (P = .011; Table 2).

**Discussion**

Essential oils containing lower levels of eucalyptol (1,8-cineole) have demonstrated anxiolytic and antidepressant effects in previous studies, making eucalyptol a potential adjunctive therapy to support safe, cost-effective management of anxiety and depression.21-23 As previous studies used essential oils containing other potentially active compounds, our study used eucalyptol (1,8-cineole, 99%) to further investigate its effect on anxiety and depression as well as potential activity at the benzodiazepine site on the GABA<sub>A</sub> receptor in the rat central nervous system.

Decreased psychomotor activity is an unwanted side effect of anxiolytic therapy. Anxiolytic medications can severely limit the patient’s ability to perform activities of daily living. Mean speed and mean time mobile were assessed to determine if eucalyptol affected psychomotor activity, and statistical significance was found for both. The eucalyptol group exhibited significantly increased mean speed and mean time mobile compared with midazolam, a known psychomotor depressant, and exhibited no significant difference compared with the vehicle. These results suggest that pure eucalyptol does not significantly affect psychomotor activity.
Research conducted by Majnooni and colleagues demonstrated similar results with essential oils containing eucalyptol (21%). The observation that mean speed and mean time mobile were significantly decreased in a comparison of midazolam alone with midazolam plus eucalyptol suggests that eucalyptol may attenuate the psychomotor effects of midazolam. These results support the findings of Quílez and colleagues that eucalyptus attenuated the psychomotor depressant effects of diazepam.

The EPM is a well-established model used in rodent research to observe for anxiety-like behavior. It is based on 2 opposing instincts in the rodent: exploring a familiar environment vs fearing an open environment. Increased time spent in the open arm indicates decreased anxiety, which was the primary variable of interest in this study. Our research found no significant difference between eucalyptol and the positive control midazolam in terms of open-arm time ratio, suggesting similar anxiolytic effects. Majnooni and colleagues demonstrated not only that the essential oil Achillea wilhelmsii, containing eucalyptol (21%), had no psychomotor effects but also that Achillea wilhelmsii exhibited anxiolytic effects. This is of particular interest, as the open-arm time ratio is the most significant measure of anxiolysis in the EPM. Our results also demonstrated a significant increase in open-arm time ratio when we compared midazolam with flumazenil plus eucalyptol. The lack of significant difference between eucalyptol and midazolam suggests that flumazenil, a known benzodiazepine antagonist, attenuated the effects of eucalyptol. Two studies have shown essential oils containing eucalyptol as having anxiolytic effects, but neither observed for effects at the benzodiazepine site on the GABAA receptor using pure eucalyptol. The eucalyptol group maintained normal psychomotor activity in terms of mean speed and mean time mobile; however, potential anxiolytic properties were identified in the open-arm time ratio. Because the flumazenil, a benzodiazepine antagonist, diminished the open-arm time on the EPM when administered with eucalyptol, it would be interesting to speculate that eucalyptol may interact at the benzodiazepine site on the GABA receptor, producing anxiolysis while maintaining normal psychomotor activity, the pharmacodynamic profile of an ideal anxiolytic.

The FST is a well-established model for evaluating behavioral despair in rodent research. Fifty-five Sprague-Dawley rats were randomly assigned to 1 of 5 groups (n = 11 per group). Each of the 5 groups received 2 intraperitoneal injections: vehicle (Veh) dimethyl sulfoxide (DMSO) plus (+) normal saline; eucalyptol (Euc) + DMSO; midazolam (Mid) + DMSO; flumazenil (Flu) + Euc; and Mid + Euc.
FPO. Our research showed no statistical significance when we compared mean time mobile of the eucalyptol group with that of the other groups. Most antidepressant medications currently on the market require a minimum of 2 to 4 weeks of administration to reach therapeutic effects. A possible reason for these results could be the 1-time intraperitoneal dose of 1mg/kg used in this study. Thus, a 1-dose regimen probably would not sufficiently maintain therapeutic plasma levels to potentially produce antidepressant effects.

Statistical significance was found between eucalyptol and the midazolam group as well as the midazolam plus eucalyptol group when we measured the rats’ FPO. The eucalyptol group demonstrated a significant increase in FPO when we measured the rats' FPO. Our research showed no statistical significance when we compared mean time mobile of the eucalyptol group and the midazolam group as well as the midazolam plus eucalyptol group when we measured the rats’ FPO. The data from the FST demonstrated eucalyptol lacked antidepressant effects with a single intraperitoneal injection of 1 mg/kg.

In this study, statistical significance was found with a single intraperitoneal dose (1 mg/kg) of eucalyptol when we measured mean speed (centimeters per second), mean time mobile (seconds), and open-arm time ratio (percentage) on the EPM. Eucalyptol (99%) demonstrated anxiolytic effects but did not completely agonize the benzodiazepine site on the GABA_A receptor. Therefore, we hypothesize there may be other receptors involved in its effects. For example, eucalyptol has been shown to exert its effects at the 5HT-3 receptor. Furthermore, future studies would take into account these different receptors to appreciate the pharmaceutical application of eucalyptol, its mechanism of action, and potential binding sites. These studies could include long-term regimens with stabilized plasma levels of eucalyptol, alternative dosing regimens, alternative routes of administration, and molecular biology receptor binding assays. These assays could interrogate and explain the type of affinity that eucalyptol may have as an agonist, partial agonist, or antagonist to various receptors’ neural modulation.

Conclusion
The results of this study suggest that eucalyptol (1,8-cineole) produces anxiolytic effects by acting at the benzodiazepine site on the GABA_A receptor while sparing psychomotor activity. However, no effects on behavioral despair were demonstrated in the FST. For a better understanding of the potential of eucalyptol as an anxiolytic or antidepressant, future studies should continue to investigate its effect at the benzodiazepine site on the GABA_A receptor and other receptors.

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